UNIVERSIDADE ESTADUAL DE LONDRINA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

ELISABETE TAKIUCHI

DIAGNÓSTICO MOLECULAR E ANÁLISE DE POLIMORFISMOS DO GENE S1 DE ESTIRPES BRASILEIRAS DO CORONAVÍRUS BOVINO EM FEZES DIARREICAS DE BEZERROS NATURALMENTE INFECTADOS

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Tese apresentada para a obtenção do título de Doutor em Ciência Animal (área de concentração em Sanidade Animal) da Universidade Estadual de Londrina.

Orientador: Prof. Dr. Amauri Alcindo Alfieri

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RESUMO

TAKIUCHI, E. **Diagnóstico molecular e análise de polimorfismos do gene S1 de estirpes brasileiras do coronavírus bovino em fezes diarreicas de bezerros naturalmente infectados.** 2006. 108f. Tese (Doutorado em Ciência Animal, área de concentração em Sanidade Animal) – Universidade Estadual de Londrina, Londrina. 2006.

O coronavírus bovino (BCoV) é um importante agente etiológico de diarréia neonatal em bezerros. Usualmente, as técnicas para a detecção do BCoV limitam-se a métodos de baixa sensibilidade como ensaios imunoenzimáticos e testes de hemaglutinação (HA)/ inibição da hemaglutinação (HI). Embora a PCR seja altamente sensível e específica, a natureza da amostra biológica é um fator limitante no diagnóstico do BCoV, devido à presença de inibidores de PCR nas fezes que podem gerar resultados falso-negativos. A utilização de um controle interno na reação de PCR tem sido útil para monitorar a qualidade da extração e amplificação do ácido nucléico. Este trabalho teve como objetivo desenvolver um sistema de Semi nested-PCR (SN-PCR) para amplificação de um fragmento de 251 pares de bases (pb) do gene do nucleocapsídeo (N) do BCoV a partir de amostras fecais congeladas (*n*=25) e frescas (*n*=25) de bezerros com sinais clínicos de diarréia e naturalmente infectados. Para aperfeiçoar a detecção do BCoV em amostras fecais pela SN-PCR foi avaliada a inclusão de um controle interno e os resultados foram comparados com uma RT-PCR convencional descrita na literatura. O gene N do BCoV foi detectado pela SN-PCR e RT-PCR em respectivamente, 24% (12/50) e 8% (4/50) das amostras analisadas (*K*=0,43). Somente as amostras que não sofreram o congelamento foram positivas na RT-PCR enquanto a SN-PCR descrita neste trabalho detectou o BCoV em ambas condições de preservação. A possibilidade de resultados falso-negativos foi descartada com a amplificação do controle interno em todas as amostras analisadas. A inclusão de um controle interno na PCR possibilitou maior precisão no diagnóstico do BCoV como agente etiológico da diarréia em bezerros. Com o objetivo de estabelecer as relações genéticas entre as três estirpes brasileiras de BCoV daquelas descritas em outros países, foi desenvolvida uma estratégia de seqüenciamento para o gene que codifica a subunidade S1 da glicoproteína da espícula aplicando posteriormente o método da máxima parcimônia para realização das estimativas filogenéticas. A análise filogenética da seqüência total de nucleotídeos e dos aminoácidos deduzidos do gene S1 revelou que as estirpes brasileiras descritas neste trabalho apresentaram maior identidade com a estirpe entérica americana BCoV-ENT com 98,7% e 98,7%, respectivamente. A menor identidade foi observada com o protótipo BCoV Mebus com 97,8% (nucleotídeos) e 96,8% (aminoácidos). Na reconstrução da árvore filogenética não enraizada, baseada na região hipervariável da subunidade S1, nossas amostras foram agrupadas com as amostras americanas (BCoV-ENT, 182NS), amostras canadenses de diarréia neonatal (BCQ20, BCQ7373, BCQ2070, BCQ9, BCQ571, BCQ1523) e uma amostra canadense de diarréia do inverno (BCQ2590) e agruparam-se em um ramo separado das estirpes de origem coreana e as estirpes respiratórias de BCoV. Observou-se ainda que as três estirpes localizaram-se em um ramo bem distante de outras amostras brasileiras (AY606193, AY606194) anteriormente descritas. Portanto, estes dados colaboram com a reconstrução genealógica dos BCoV e sugerem a existência de no mínimo dois BCoV circulantes no Brasil. Adicionalmente o presente trabalho apresenta a descrição de uma mutação inédita na seqüência de aminoácidos observada no sítio de clivagem da proteína S. As prováveis conseqüências biológicas desta mutação foram especuladas a partir de estudos relacionados com o coronavirus da hepatite dos comundongos (MHV).

Palavras-chave: BCoV. Diarréia neonatal. Diagnóstico. RT-PCR. Variabilidade Genética

ABSTRACT

TAKIUCHI, E. **Molecular diagnosis and polymorphisms analysis of the S1 gene of Brazilian strains of bovine coronavirus in diarrheic feces from calves naturally infected.** 2006. 108f. Thesis (Doctorate Degree in Animal Science) - Universidade Estadual de Londrina, Londrina. 2006.

Bovine coronavirus (BCoV) is an important infectious agent of neonatal diarrhea in calves worldwide. Currently, the routine detection and diagnosis of BCoV have been mainly dependent on assays with low sensitive as the enzyme-linked immunosorbent assay or hemagglutination (HA) / hemagglutination inhibition (HI) tests. Although PCR has been described as an assay with high sensitivity and specificity, the feces remains the most difficult clinical specimen for nucleic acid extraction and amplification due to the presence of PCR inhibitors that may yield false negative results. The use of an internal control in the PCR reaction has been most commonly applied to monitor and evaluate these failures. The aim of the present study was to develop and evaluate a semi-nested PCR (SN-PCR) to amplify a 251 base pairs (bp) fragment of BCoV N gene from fresh (*n*=25) and frozen (*n*=25) diarrheic fecal samples of naturally infected calves. To improve detection of BCoV in fecal samples by the SN-PCR an internal control was developed, and the results were compared with a conventional RT-PCR assay previously described in the literature. The rates of positive samples by SN-PCR and RT-PCR were respectively, 24% (12/50) and 8% (4/50) ((*K*=0,43). Only fresh samples were positive in RT-PCR while the SN-PCR detected BCoV in both storage conditions. The possibility of false negative results was excluded due to the internal control amplification in all samples analysed. The inclusion of an internal control provided more accurate diagnosis of BCoV as causative agent of diarrhea in calves. To verify genetic relationships among the three Brazilian strains of BCoV from other BCoV strains from different countries, was developed a sequencing strategy for the gene which codes spike glycoprotein S1 subunit and further phylogenetic analysis using maximum parsimony. The phylogenetic analysis of the entire S1 nucleotide and deduced amino acid sequences showed that BCoV Brazilian strains described in this study were more similar to the American enteric strain BCoV-ENT with 98.7% and 98.7%, respectively. The lower identity was observed with the BCoV Mebus strain with 97.8% (nucleotide level) and 96.8% (amino acid level). The phylogenetic unrooted tree based on hipervariable region of the S1 subunit, showed our strains clustered with American strains (BCoV-ENT, 182NS), Canadian calf diarrhea strains (BCQ20, BCQ7373, BCQ2070, BCQ9, BCQ571, BCQ1523) and an Canadian winter dysentery strain (BCQ2590) but clustered on a separate branch of the Korean and respiratory BCoV strains. Besides, it was described that BCoV strains of this study clustered in a separate branch of the previously published Brazilian strains (AY606193, AY606194). Therefore, these data corroborate to the genealogical construction and suggest the existence of at least two different BCoV strains circulating in Brazil. The present study also relates an exclusive mutation in the amino acid sequence of the S protein cleavage site. The probable biological effects of this mutation are discussed based on murine hepatitis coronaviruses (MHV) studies.

Key Words: BCoV. Neonatal diarrhea. Diagnosis. RT-PCR. Genetic Variability

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1. REVISÃO DE LITERATURA

1. REVISÃO DE LITERATURA

Os coronavírus compreendem um gênero da família *Coronaviridae,* da ordem *Nidovirales,* cujas espécies estão organizadas em três grupos (1, 2 e 3) distintos, de acordo com as propriedades antigênicas e moleculares (Gonzáles et al., 2003). O termo "coronavírus" é derivado do latim *corona* devido à aparência das glicoproteínas da superfície do virion, em forma de "coroa", à microscopia eletrônica (Tyrrel et al., 1968).

O grupo 1 de coronavírus inclui: vírus da gastroenterite transmissível dos suínos (TGEV); coronavírus felino (FCoV); vírus da peritonite infecciosa felina (FIPV); coronavírus canino (CCoV); coronavírus humano 229E (HCoV-229E); coronavírus respiratório suíno (PRCoV); e vírus da diarréia epidêmica suína (PEDV). O grupo 2 inclui outros patógenos de importância veterinária tais como o coronavírus bovino (BCoV) e o vírus da encefalomielite hemaglutinante suína (HEV), bem como coronavírus humano OC43 (HCoV-OC43). Coronavírus que infectam ratos e camundongos também pertencem ao grupo 2 como o vírus da sialodacrioadenite dos ratos (SDAV), coronavírus dos ratos (RtCoV) e o vírus da hepatite dos camundongos (MHV). O grupo 3 é formado pelos coronavírus aviários, entre eles, o vírus da bronquite infecciosa das galinhas (IBV), coronavírus do peru (TCoV) e dos faisões (PhCoV) (Lai e Holmes, 2001; Cavanagh et al., 2002).

Em novembro de 2002 foi identificado em seres humanos o coronavírus da síndrome respiratória aguda grave (SARS-CoV), cuja análise do genoma total tem demonstrado divergência filogenética em relação aos três grupos antigênicos já conhecidos (Ksiazek et al., 2003). Entretanto, a análise do gene da polimerase tem indicado que o SARS-CoV possa ser um membro distante do grupo 2 (Snijder et al., 2003). De acordo com o Comitê Internacional de Taxonomia dos Vírus (ICTV) o SARS-CoV é, atualmente, classificado no grupo 2 dos coronavírus. (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm)

Morfologicamente, os coronavírus apresentam-se à microscopia eletrônica envoltos por um envelope glicoproteico com diâmetro aproximado de 100-120 nm. O genoma consiste uma fita simples de RNA de polaridade positiva, cujo tamanho pode variar de 27 a 32 kb, que o torna o mais longo de todos os vírus conhecidos que possuem RNA genômico (Lai e Cavanagh, 1997).

Todos os coronavírus possuem estrutura genômica semelhante, organizada em 7 a 10 janelas de leitura aberta (ORF) de acordo com o grupo antigênico ao qual pertence (Fig.1). O primeiro segmento genômico abrange cerca de dois terços da extremidade 5' do genoma, e compreende duas ORFs sobrepostas (ORF1a e ORF1b). Estas ORFs são traduzidas em uma poliproteína, que é o precursor da polimerase viral. No terço genômico restante estão organizados quatro genes codificadores de proteínas estruturais que são comuns em todas as espécies, na seguinte ordem: glicoproteína da espícula (S), proteína do envelope (E), proteína de membrana (M) e a proteína do nucleocapsídeo (N). Algumas espécies que constituem o grupo 2 dos coronavírus, como o BCoV e MHV, possuem adicionalmente uma glicoproteína de membrana denominada hemaglutinina esterase (HE) e a proteína interna (I) (Fischer et al., 1997; Senanayake e Brian, 1997). Adicionalmente, estes genes intercalam-se com variadas ORFs codificantes de proteínas não-estruturais, que diferem em número, seqüência de nucleotídeos, organização e expressão entre os coronavírus (Lai e Cavanagh, 1997) (Fig.1).

Durante a replicação citoplasmática dos coronavírus, ocorre a síntese de uma fita genômica completa de polaridade negativa que servirá como molde para a síntese do RNA genômico de polaridade positiva. Entretanto, também são sintetizados múltiplos RNAs subgenômicos que atuarão como RNAm das proteínas virais. Experimentos com células infectadas pelo coronavírus demonstraram diferença considerável no número de RNAs

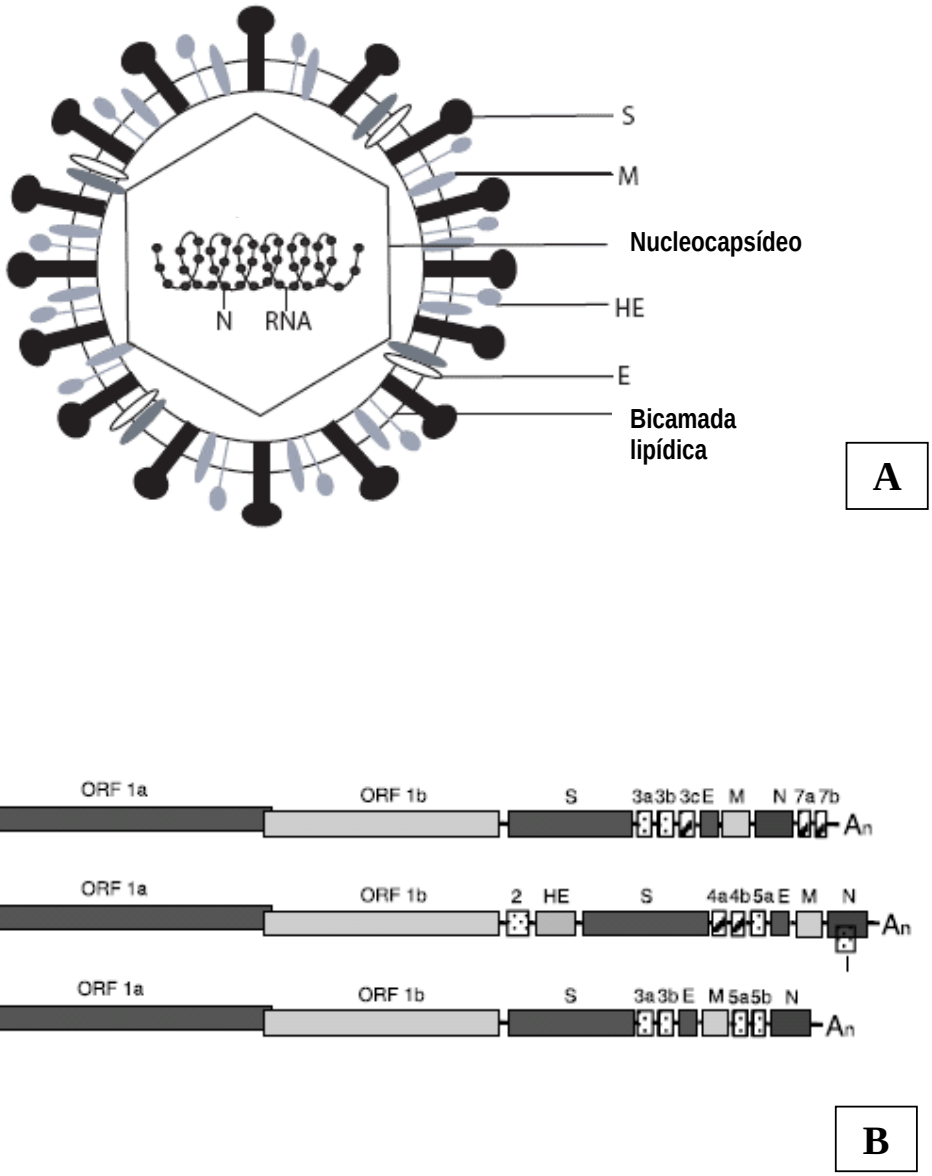

Fig.1. Modelo estrutural do coronavírus. **A:** diagrama esquemático da estrutura do virion. S, glicoproteína da espícula; M, proteína da membrana; E, proteína do envelope; HE, glicoproteína HE; N, proteína do nucleocapsídeo. **B:** Estrutura genômica dos diferentes grupos de coronavírus: organização dos genes codificadores de proteínas estruturais e não-estruturais.
Fonte: Lai e Holmes (2001); Gonzáles et al. (2003)

subgenômicos transcritos entre os genes codificantes de proteínas estruturais (Spaan et al., 1983).

A proteína N é uma fosfoproteína que associada ao RNA genômico forma o nucleocapsídeo helicoidal (Lapps et al., 1987; Clark, 1993). Além de desempenhar função estrutural, a proteína N também participa nos processos de transcrição e replicação viral (Compton et al., 1987). O gene que codifica a proteína N é altamente conservado entre os isolados da mesma espécie e o número de RNAs subgenômicos transcritos para essa proteína é significativamente maior nas células infectadas pelos coronavírus quando comparado às demais proteínas estruturais (Hiscox et al., 2001).

A proteína M é a proteína mais abundante do virion sendo atribuída a ela funções de montagem da partícula viral e de interação vírus-hospedeiro. Uma das principais funções da proteína M é direcionar a incorporação da glicoproteína S e da proteína N no interior do virion (Narayanan et al., 2000).

A proteína E é uma proteína integral de membrana, que juntamente com a proteína M, participa da montagem da estrutura viral. Para algumas espécies de coronavírus, as células que expressam somente as proteínas M e E têm demonstrado serem suficientes para a formação de estruturas semelhantes às partículas virais (de Haan et al., 1998). A capacidade de induzir apoptose em células infectadas também tem sido atribuída à proteína E (An et al., 1999).

A glicoproteína HE é encontrada em alguns coronavírus do grupo 2 e no TCoV (grupo 3) como pequenos peplômeros na superfície do virion. A proteína HE de vários coronavírus, inclusive o BCoV, se liga a resíduos 9-O-acetil de ácidos siálicos, contribuindo com as atividades de hemaglutinação e hemadsorção dos coronavirus. Essa proteína apresenta certa similaridade funcional com a proteína HE do vírus Influenza C, uma vez que possui

atividade esterase na destruição de receptores contendo resíduos 9-O-acetil de ácidos siálicos (Schultze et al., 1991a).

Outra particularidade encontrada no genoma de alguns coronavírus representantes do grupo 2, tais como BCoV e MHV, é a existência de uma proteína estrutural interna (proteína I) inserida no gene do nucleocapsídeo. Estudos conduzidos com o MHV têm demonstrado que a proteína I não é essencial para a replicação do vírus *in vitro* e tampouco como determinante patogênico em camundongos (Lapps et al., 1987; Fischer et al., 1997).

A glicoproteína S dos coronavírus desempenha importantes funções na patogênese viral incluindo a interação inicial do vírus à célula hospedeira, disseminação viral célula a célula e como determinante de tropismo tecidual. A interação inicial do vírus ocorre por meio da ligação da proteína da espícula com receptores celulares específicos. A capacidade dos coronavírus se replicarem em determinadas linhagens celulares depende, exclusivamente, da sua capacidade de interação com o receptor celular (Cavanagh, 1995). Vários receptores celulares já foram identificados incluindo a aminopeptidase N (APN) e a molécula de adesão célula-antígeno carcinoembriônico (CEACAM) que são utilizados pelos coronavírus do grupo 1 e pelo MHV, respectivamente (Yeager et al., 1992; Dveksler et al., 1993). Os coronavirus do grupo 2, tais como BCoV, HCoV-OC43 e PHEV, se ligam a receptores contendo resíduos 9-O-acetil de ácidos siálicos (Schultze et al., 1991b; Krempl et al., 1995). Em decorrência da alta afinidade pelos resíduos 9-O-acetil de ácidos siálicos a glicoproteína S do BCoV possui intensa atividade hemaglutinante. Embora também seja reconhecida a atividade hemaglutinante da glicoproteína HE, experimentos têm demonstrado que a glicoproteína S é uma hemaglutinina muito mais potente do que a HE (Schultze et al., 1991b).

Após a maturação no complexo de Golgi, a proteína S é clivada em duas subunidades, S1 e S2. A subunidade S1 contém um domínio de ligação ao receptor celular (RBD), bem como uma região hipervariável (HVR). A subunidade S2 é altamente conservada, contendo

características comuns de muitas proteínas virais de fusão, incluindo dois domínios HR1 e HR2 (do inglês *heptad repeat*) bem como um domínio transmembrana. Acredita-se que esses domínios sejam importantes para a entrada do vírus na célula e no processo de fusão célula-célula (Weiss e Navas-Martin, 2005).

O RBD está localizado em regiões distintas para cada coronavírus e variações encontradas neste domínio têm demonstrado alterar o tropismo e a patogênese viral. No caso do MHV, o RBD é representado pelos 330 primeiros aminoácidos da subunidade S1. Os RBDs do HCoV-229E (resíduos 417 a 547) e SARS-CoV (resíduos 318 a 510) são também encontrados na S1, embora não na região amino-terminal como ocorre com o MHV. Entretanto, a localização exata do RBD do BCoV ainda não foi determinada (Kubo et al., 1994; Bonavia et al., 2003; Wong et al., 2004).

A região hipervariável (HVR) é outro domínio localizado na subunidade S1 e tem sido objeto de estudos em alguns coronavírus. A HVR do MHV está situada logo após a região do RBD e mutações encontradas nesse domínio também têm sido relacionadas às alterações na patogênese e no tropismo viral (Weiss e Navas-Martin, 2005).

São atribuídas aos coronavírus doenças respiratórias crônicas e agudas, infecções entéricas, hepáticas e do sistema nervoso central (Weiss e Navas-Martin, 2005). O BCoV, representado por um único sorotipo, ocasiona infecções entérica e respiratória em bezerros que podem resultar em impacto econômico importante à atividade pecuária. O quadro clínico conhecido como disenteria do inverno (*winter dysentery*) em vacas adultas também tem sido atribuído ao BCoV, ocasionando drástica redução na produção de leite e/ou morte do animal (Benfield e Saif, 1990).

Embora a etiologia das diarréias neonatais seja bastante complexa e multifatorial, entre as causas infecciosas de origem viral o rotavírus e o coronavírus estão entre os patógenos mais frequentemente diagnosticados durante os episódios de diarréia em bezerros com até 60

dias de idade, estando presentes em aproximadamente 60% dos casos (Janke, 1989; Jerez, 1997; Alfieri et al., 2006).

A intensidade dos sinais clínicos decorrentes da infecção pelo BCoV está relacionada com a idade do animal, *status* imunológico, quantidade de vírus infectante e a estirpe viral envolvida. Os bezerros mais jovens e aqueles com falha de proteção passiva apresentam diarréia mais intensa e de curso agudo, indistinguível da diarréia associada às infecções pelo rotavírus bovino (Clark, 1993).

A eletromicroscopia é uma técnica de diagnóstico utilizada para a identificação direta das partículas de coronavírus em fezes diarréicas (Bulgin et al., 1989). Contudo, a presença comum de estruturas membranosas pleomórficas no extrato fecal, semelhantes aos coronavírus, pode dificultar a sua identificação (Saif, 1990).

O isolamento viral em cultivo celular é considerado o método padrão ouro para a detecção de muitos vírus. A adaptação do BCoV em culturas celulares tem sido obtida com a utilização da linhagem HRT 18, derivada de um adenocarcinoma retal humano (Tsunemitsu et al., 1991). Entretanto, devido à dificuldade na amplificação viral, o isolamento não é aplicado rotineiramente para o diagnóstico das diarréias neonatais bovinas ocasionadas pelo BCoV. A identificação de efeito citopático é dependente da presença de enzimas proteolíticas no meio de cultura, como a tripsina (Kapil et al., 1996). Adicionalmente, o isolamento viral é uma técnica laboriosa, de custo elevado e dispendiosa em relação ao tempo dedicado.

Outros métodos de diagnóstico são elaborados com base na propriedade hemaglutinante do vírus, tais como os testes de hemaglutinação (HA) e de inibição da hemaglutinação (HI). O HA, quando realizado diretamente em amostras fecais, deve ser interpretado com cautela uma vez que o extrato fecal pode conter hemaglutininas não-específicas responsáveis por resultados falso-positivos. A presença de imunoglobulina A (IgA) secretória no intestino também pode mascarar o resultado. O método de HI, a partir de

amostras de soro, pode ser efetuado simultaneamente à reação de hemaglutinação para aumentar a especificidade do teste. Contudo, a presença de imunoglobulinas séricas adquiridas passivamente pelo colostro pode interferir no diagnóstico. A técnica de HI utilizando soro hiperimune também pode ser utilizada com o objetivo de aumentar a especificidade do HA em amostras fecais positivas (Sato et al., 1977).

Os testes imunoenzimáticos como o ELISA e a imunoistoquímica, por apresentarem baixa sensibilidade para o BCoV, não são rotineiramente aplicados no diagnóstico direto. São descritos sistemas de ELISA com anticorpos de captura monoclonais e policlonais para determinantes antigênicos do BCoV. Entretanto, devido à baixa afinidade dos complexos antígeno-anticorpo, estes tendem a se dissociar durante as múltiplas lavagens que envolvem o processo. A acurácia do ELISA também pode ser comprometida pelo anticorpo colostral consumido pelo bezerro, que resultará na formação de complexos antígeno-anticorpo, e conseqüente perda da reatividade do antígeno. A imunoistoquímica tem seu uso limitado aos animais que evoluem para o óbito ou que possam ser sacrificados (Zhang et al., 1997; Schoenthaler e Kapil, 1999).

Embora a participação do BCoV como causador de surtos de diarréia neonatal em bezerros esteja bem definida, poucos estudos sobre a prevalência dessa virose são realizados, principalmente devido às dificuldades encontradas no processo diagnóstico. Nas fases precoce e tardia da infecção os animais eliminam baixos títulos do vírus. Portanto, para estimar a real prevalência da infecção em rebanhos, ou em determinada região, é obrigatória a utilização de técnicas de diagnóstico que apresentem alta sensibilidade e também especificidade.

A utilização de métodos de diagnóstico que baseiam-se na amplificação de uma seqüência conservada do RNA viral, especificamente a transcrição reversa seguida pela reação em cadeia da polimerase (RT-PCR), tem sido eficiente na detecção do BCoV. Tsunemitsu et al. (1999) descreveram que a RT-PCR apresentou melhor sensibilidade quando

comparada às técnicas de ELISA e imunomicroscopia eletrônica. De forma semelhante, Cho et al. (2001) relatam que a RT-PCR e a Nested-PCR (N-PCR) foram 50 e 5.000 vezes mais sensíveis que o teste de ELISA, respectivamente.

Embora a RT-PCR e a N-PCR tenham demonstrado eficiência no diagnóstico do BCoV, fatores limitantes relacionados à natureza da amostra biológica devem ser avaliados. A presença de inibidores da enzima Taq DNA Polimerase no extrato fecal constitui-se o maior inconveniente na padronização das técnicas moleculares de diagnóstico (Wilson, 1997). Para controlar essas possíveis falhas, a otimização de métodos de extração de ácidos nucléicos e o uso associado de um controle interno nas reações de amplificação é apropriadamente indicado (Dingle et al., 2004; Hoorfar et al., 2004). Entretanto, essa ferramenta de controle não tem sido rotineiramente padronizada e utilizada para o diagnóstico de BCoV.

Outro problema inerente ao vírus que pode interferir negativamente no diagnóstico é o extenso RNA genômico, com aproximadamente 30 kb, que favorece a formação de estruturas secundárias e impede que as cópias de cDNA sejam eficientemente transcritas. Além disso, assim como em todos os vírus que possuem RNA genômico, a freqüência de mutação é muito maior do que em vírus DNA, devido à falta de atividade de correção das polimerases dependentes de RNA. Com uma estimativa de um erro para 10.000 bases, a RNA polimerase do coronavírus pode gerar cerca de 3 mutações randômicas a cada 30 kb de genoma produzido em uma célula infectada (Malpica et al., 2002).

Portanto, torna-se evidente que a seleção de uma região altamente conservada no genoma viral bem como a otimização da técnica de PCR são igualmente importantes para o aumento da freqüência de diagnóstico do BCoV. O gene que codifica a proteína N é altamente conservado entre as estirpes do BCoV e também é o mais abundante nas células infectadas na forma de RNA subgenômico. Desta forma, a característica conservada do gene N aliada à

maior disponibilidade nas células infectadas, favorece significativamente a sensibilidade da técnica.

Contrariamente aos estudos de prevalência, os estudos relacionados à variabilidade genética entre os BCoV devem abranger regiões genômicas mais polimórficas para permitir a diferenciação entre as estirpes de uma mesma espécie de coronavírus. A filogenia molecular é o estudo das relações evolutivas entre os organismos utilizando dados obtidos a partir de técnicas de biologia molecular. Usando modelos probabilísticos, Wu e Yan (2005) relatam que a glicoproteína S é a mais sensível às mutações entre as proteínas do coronavírus. Desta forma, os estudos que abrangem as relações filogenéticas entre as estirpes de BCoV convergem primariamente na seqüência genômica que codifica a proteína S, seja na sua totalidade, ou parcialmente, pela seleção de domínios que apresentam maior variabilidade, como por exemplo a região HV da subunidade S1 (Chouljenko et al., 2001; Hasoksuz et al., 2002; Jeong et al., 2005, Brandão et al., 2006; Liu et al., 2006).

Existe uma série de algoritmos que podem ser aplicados em estimativas filogenéticas. Entre esses algoritmos, o método da máxima parcimônia (MP) utiliza critérios qualitativos para as inferências filogenéticas e tem sido amplamente aplicado em virologia. A MP baseia-se na suposição de que a árvore mais provável é a que requer menor número de mudanças para explicar toda a variação observada na matriz de caracteres (Brinkman e Leipe, 2001; Miyaki et al., 2001).

A glicoproteína S do BCoV é uma proteína com aproximadamente 151 kDa composta por 1.363 aminoácidos (Abraham et al., 1990, Zhang et al., 1991). Após a glicosilação, a proteína S é clivada por proteases com atividade semelhante à tripsina em duas subunidades: S1 (N-terminal) e S2 (C-terminal). Das duas subunidades geradas após a clivagem protéica, a S2 é altamente conservada entre os BCoV, sendo que variações são muito mais freqüentes no fragmento S1 (Gallagher e Buchmeier, 2001). A S1, que forma a porção bulbar da proteína S,

se liga a receptores específicos na membrana celular e, portanto, está mais exposta aos anticorpos neutralizantes. A sequência de aminoácidos (aa) básicos KRRSRR, que abrange os aa 763 a 768, constitui o sinal de clivagem da proteína e também é altamente conservada entre os BCoV. O ponto de clivagem ocorre entre os aa 768 e 769 (Storz et al., 1981).

Embora a função da glicoproteína S nas diferentes estirpes do BCoV seja desconhecida, estudos relacionados ao MHV, o membro da família *Coronaviridae* mais estudado e também pertencente ao grupo 2, tem mostrado que esse evento não é essencial para o início da infecção, mas está associado com a maior capacidade de fusão celular (sincício) nas células infectadas. Entretanto, mutações no sinal de clivagem de estirpes do MHV determinaram a perda da clivagem e, conseqüentemente, incapacidade de formação de sincícios (Yamada et al., 1997).

Estudos mais recentes demonstraram que a mutação de um único aa no sítio de clivagem de MHV foi capaz de determinar aumento significativo da carga viral e maior disseminação tecidual (Navas-Martin et al., 2005).

Na América do Sul, são extremamente escassos os estudos epidemiológicos que determinam a freqüência do BCoV. Entretanto, a utilização de técnicas de diagnóstico sensíveis como a RT-PCR é fundamental para não subestimar a real prevalência do BCoV. Adicionalmente, os estudos filogenéticos baseados em regiões polimórficas da proteína S1 podem fornecer dados relevantes para o entendimento da variabilidade e a relação entre as estirpes de BCoV provenientes de diferentes regiões geográficas.

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2. OBJETIVOS

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2.1. Objetivo geral

- Desenvolver e avaliar sistemas de RT-PCR para o diagnóstico e caracterização molecular do BCoV em amostras de fezes diarréicas de bezerros naturalmente infectados.

2.2. Objetivos específicos

- Desenvolver e avaliar uma semi-nested PCR, com controle interno da reação, para a detecção simultânea do gene do nucleocapsídeo do BCoV e do gene ND5 do DNA mitocondrial bovino em amostras de fezes de bezerros com e sem sinal clínico de diarréia;

- Comparar os resultados obtidos com a semi-nested PCR e controle interno com um sistema de RT-PCR convencional descrito na literatura para a detecção do gene N do BCoV em amostras de fezes sob diferentes condições de estocagem;

- Desenvolver uma estratégia de seqüenciamento por sobreposição para o gene S1 do BCoV diretamente das amostras clínicas e estabelecer as relações filogenéticas entre as estirpes brasileiras do BCoV com outros BCoV identificados em diferentes regiões geográficas;

- Identificar e avaliar as mutações genômicas exclusivas da subunidade S1 das estirpes brasileiras de BCoV.

3. ARTIGO PUBLICADO

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Improved detection of bovine coronavirus N gene in faeces of calves infected naturally by a semi-nested PCR assay and an internal control

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Abstract

Bovine coronavirus (BCoV), a positive sense single-stranded RNA virus, is an important causative agent of neonatal diarrhoea in calves from beef and dairy cattle worldwide. The routine detection and diagnosis of BCoV have been mainly dependent on assays with low sensitivity. The aim of the present study was to develop and evaluate a semi-nested PCR (SN-PCR) to amplify a 251 bp fragment of BCoV N gene from fresh ($n = 25$) and frozen ($n = 25$) diarrhoeic faecal samples of naturally infected calves. To improve detection of BCoV in faecal samples by the SN-PCR an internal control was developed, and the results were compared with a conventional RT-PCR assay. The rates of positive samples by SN-PCR and RT-PCR were 24% (12/50) and 8% (4/50), respectively ($K = 0.43$). Only fresh samples were positive in RT-PCR while the SN-PCR detected BCoV in both fresh and frozen faecal samples. The sensitivity of SN-PCR was determined by 10-fold serial dilutions of the BCoV Kakegawa strain (HA titre: 256) that was detected until 10^{-7} dilution. The specificity of the amplicons was assessed by restriction fragment length polymorphism and sequence analysis. The inclusion of an internal control provides a way to detect assay inhibition in faecal samples and failure of nucleic acid extraction that allow reduction of the number of false-negative results.

Keywords: Calves; Diarrhoea; Bovine coronavirus; SN-PCR

1. Introduction

The neonatal diarrhoea complex is one of the main causes of calf morbidity and mortality causing major economic losses in many dairy and beef herds. Diarrhoea in calves is considered a multifactorial disease. Several environmental, managemental, nutritional and physiological factors may occur either alone or in synergy with the different infectious agents such as enterotoxigenic *Escherichia coli*, *Cryptosporidium* sp., bovine group A rotavirus, calicivirus, torovirus and bovine coronavirus (BCoV) (Snodgrass et al., 1986; Duckmanton et al., 1998; Naciri et al., 1999; Van der Poel et al., 2000). This emphasizes the difficulties in investigating and diagnosing the infectious aetiology of calf diarrhoea. Diarrhoea caused by BCoV is clinically important and the virus also infects the respiratory tract of cattle and has been associated with winter dysentery in adult cattle (Clark, 1993; Saif, 1990).

BCoV is a member of the order *Nidovirales*, *Coronaviridae* family, that possesses a single-stranded, non-segmented RNA genome of positive polarity. The genome of coronaviruses is the largest among the RNA viruses, 26–30 kb long, and encodes a nested set of multiple subgenomic mRNAs during infection (Lai and Cavanagh, 1997). The virion contains five major structural proteins: the nucleocapsid protein (N), the transmembrane protein (M), the hemaglutinin esterase protein (HE), the spike protein (S) and the small membrane protein (E). The N protein is a phosphoprotein of 50–60 kd that interacts with genomic RNA to form the viral nucleocapsid and may play a role in replication of viral RNA (Lapps et al., 1987; Clark, 1993).

Electron microscopy (EM) may be used for the direct detection of BCoV in faecal samples (Collins et al., 1987; Bulgin et al., 1989). However, due to the common presence of pleomorphic membranous structures similar to the coronavirus it may be difficult to recognize BCoV by EM (Saif, 1990). Virus isolation in human rectal adenocarcinoma (HRT-18) cells is rarely used as a means of diagnosis because BCoV is difficult to isolate. Furthermore, virus isolation is a laborious method and requires

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a long time to obtain conclusive results (Dea et al., 1980; Tahir et al., 1995; Kapil et al., 1996). The haemagglutination (HA) test, performed directly with the faecal samples must be carefully interpreted since the faeces contain non-specific haemagglutinins providing false-positive results. The presence of colostral IgG1 and local IgA in faeces may also interfere with the HA results. The haemagglutination inhibition (HI) test is performed concurrently to improve the specificity of the HA test. Besides, another disadvantage of the HA/HI test is the need to maintain mouse colonies as sources of the fresh erythrocytes that are required for this diagnostic test (Sato et al., 1977; Kapil et al., 1999). Enzyme-linked immunosorbent assays (ELISA) are probably the most widely used diagnostic test for BCoV although this test lacks sensitivity (Sato and Akashi, 1993; Tsunemitsu and Saif, 1995; Schoenthaler and Kapil, 1999; Silva et al., 1999).

More sensitive tests are required to detect BCoV especially from faecal samples of calves at early or late stages of disease when they have low levels of viral shedding. Specificity is equally important to avoid false positive results.

Although BCoV has been established as an agent of diarrhoea in calves, its prevalence has been investigated to only a limited extend because of the diagnostic difficulties. However, more sensitive and specific tests for BCoV detection will permit more conclusive and larger epidemiological studies.

A reverse transcription-polymerase chain reaction (RT-PCR) assay for amplification of BCoV RNA from faecal samples has been described and its sensitivity and specificity has been reported although the detection was performed only in clinical samples from experimentally inoculated calves (Tsunemtitsu et al., 1999; Cho et al., 2001). Studies that describe the development of RT-PCR for BCoV detection from naturally infected diarrhoeic calves are still sporadic. Besides, there are no comparative studies on the ability of different primer sets to detect BCoV in clinical samples. Also, faeces remains the most difficult clinical sample for nucleic acid extraction and amplification due to the presence of PCR inhibitors that may yield false negative results (Monteiro et al., 1997). The use of an internal control in the PCR reaction has been most commonly applied to monitor and evaluate these failures, but this approach has not been used in assays for BCoV detection (Beld et al., 2004; Dingle et al., 2004; Hoorfar et al., 2004).

In this study we have developed a semi-nested PCR assay (SN-PCR) with an internal control to detect BCoV in faecal samples from naturally infected calves and have compared it with a RT-PCR assay previously described.

2. Materials and methods

2.1. Virus and cells

The HRT-18 cells were grown in Dulbecco's Modified Eagle's Medium (D-MEM, Gibco BRL, USA), supplemented with 10% fetal bovine serum (FBS, Gibco BRL, USA), 55 μg/ml gentamicine (Sigma Co., USA) and 2.5 μg/ml amphotericin B (Sigma Co., USA). The Kakegawa strain of BCoV was propagated in HRT-18 cells cultured in FBS free D-MEM and used in all standardization procedures of the RT-PCR and SN-PCR assays.

2.2. Clinical samples

Faecal samples from beef and dairy cattle herds were obtained from 50 calves up to 30 days old with signs of diarrhoea. Of the 50 faecal specimens 25 were stored at −20 °C for 2 years and the 25 remaining samples were fresh samples without previous freezing. Another 15 faecal samples from asymptomatic calves were included in this study as a control group. The samples were prepared either as 10% (w/v) suspensions of solid (control) or semi-solid faeces in 0.01 M phosphate-buffered saline (PBS) pH 7.2 (137 mM NaCl; 3 mM KCl; 8 mM Na_2HPO_4; 15 mM KH_2PO_4) or as 50% (v/v) suspensions of liquid faeces in 0.01 M PBS and centrifuged at 3000 × *g* for 15 min at 4 °C. The supernatants were used for RNA extraction.

2.3. RNA extraction

Aliquots of 400 μl from faecal suspensions were treated with SDS at a final concentration of 1% (v/v), homogenized by vortexing and kept at 56 °C for 30 min. For RNA extraction a combination of phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate methods was performed according to Barreiros et al. (2004) with slight modifications. Briefly, 400 μl of phenol/chloroform/isoamyl alcohol (25:24:1) were added, vortexed and heated at 56 °C for 15 min (Sambrook et al., 1989). The mixture was centrifuged at 10,000 × *g* for 10 min and the supernatant was transferred into a new tube and processed by the silica/guanidinium isothiocyanate method (Boom et al., 1990). The RNA was eluted from the silica pellet with 50 μl of ultrapure (MilliQ®) sterile water by 15 min incubation at 56 °C and centrifugation at 10,000 × *g* for 10 min. The supernatant fraction was kept at −20 °C until use. Aliquots of ultrapure sterile water were included as negative controls in all the RNA extraction procedures.

2.4. RT-PCR

The RT-PCR was performed using the oligonucleotide primers upstream 5′-GCCGATCAGTCCGACCAATC-3′ (nt 79–98) and downstream 5′-AGAATGTCAGCCGGGGTAT-3′ (nt 467–485) that amplify a 407 base pair (bp) fragment of the N gene of BCoV. The technique was carried out as described by Tsunemtitsu et al. (1999).

2.5. SN-PCR

For the SN-PCR assay we designed another primer set to detect BCoV from faecal samples. Nucleotide sequence data of BCoV strains were obtained from Entrez database (http://www.ncbi.nlm.nih.gov/Entrez/). For the design, sequence alignment and the preliminary evaluation of the primers used in this assay and for the restriction endonucleases selection used in the evaluation of the amplicon specificity the following softwares were used: Gene Runner Version 3.05 (Hastings Software

Inc., Hastings, NY) (http://www.generunner.com/), CLUSTAL W Multiple Sequence Alignment Program (http://www.ebi.ac.uk/clustalw/) (Thompson et al., 1997), and BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The oligonucleotide primers were designed from the highly conserved region of the N gene of the Mebus strain (GenBank accession number U00735). The sequence of primers (positions calculated from the start codon of the N gene) were: BCoV1 sense: 5′-CGATGAGGCTATTCCGAC-3′ (nt 504–521) and BCoV2 antisense: 5′-TGTGGGTGCGAGTTCTGC-3′ (nt 940–957) for the PCR reaction; BCoV3 sense: 5′-TTGCTAGTCTTGTTCTGGC-3′ (nt 707–725) and BCoV2 antisense for the SN-PCR reaction. The predicted PCR and SN-PCR products were 454 and 251 bp, respectively.

To check the efficiency of the nucleic acid extraction and amplification an internal control (BOV1 and BOV2 primers) was included for each reaction. BOV1: 5′-ATACGCCTTCATTACCAG-3′ (nt 12,231–12,248) and BOV2: 5′-TTGAATGGAGTAGTGCTG-3′ (nt 12,839–12,856) primers amplified a 626 bp fragment of the ND5 gene present in the bovine mitochondrial DNA (GenBank accession number NC_001567) (Wosiacki et al., 2005). The internal control was used in an individual reaction or in Multiplex-PCR together with BCoV1 sense and BCoV2 antisense primers in the first round of the reaction (PCR).

The reverse transcription (RT) reaction was performed with 5 μl of extracted RNA and 1 μl of the primer BCoV2 antisense (20 pmol) that was incubated at 97 °C for 4 min. Subsequently, it was placed on ice for 5 min and 10 μl of RT mix containing 1× RT buffer (50 mM Tris–HCl, pH 8.3, 3 mM $MgCl_2$, 75 mM KCl), 0.1 mM of each dNTP (Invitrogen™ Life Technologies, USA), 10 mM DTT, 100 units of M-MLV Reverse Transcriptase (Invitrogen™ Life Technologies, USA) and ultrapure sterile water to a final volume of 20 μl were added and incubated at 42 °C for 30 min and followed by inactivation at 95 °C for 5 min.

For the first round of amplification, 8 μl of the RT reaction were added to 42 μl of the PCR mix consisting of 1.5× PCR buffer (30 mM Tris–HCl pH 8.4 and 75 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μl (20 pmol) of each primer (BCoV1 sense/BCoV2 antisense and BOV1/BOV2 in single or multiplex reaction), 2.5 units *Platinum Taq* DNA polymerase (Invitrogen™ Life Technologies, USA) and ultrapure sterile water to a final volume of 50 μl. The reaction was performed in a thermocycler (PTC-200, MJ Research Co. Water Town, MA, USA) with the following time and temperature conditions: one step of 4 min/94 °C; followed by 40 cycles of 1 min/94 °C, 1 min/55 °C, 1 min/72 °C and a final step of 7 min/72 °C.

For the second round of amplification (SN-PCR) 3 μl of the first amplification product (PCR assay) were added to 47 μl of the SN-PCR mix containing 1× PCR buffer (20 mM Tris–HCl pH 8.4 and 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μl (20 pmol) of each SN-PCR primer (BCoV3 sense and BCoV2 antisense), 2.5 units of *Platinum Taq* DNA polymerase and ultrapure sterile water to a final volume of 50 μl. The reaction was performed with the following time and temperature conditions: one step of 4 min/94 °C; followed by 30 cycles of 1 min/94 °C, 1 min/55 °C, 1 min/72 °C and a final step of 7 min/72 °C.

2.6. Analysis of RT-PCR and SN-PCR

The products were analyzed by electrophoresis in a 2% agarose gel in TBE buffer pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA), ethidium bromide (0.5 μg/ml) stained and visualized under UV light.

2.7. Restriction analysis

The specificity of the SN-PCR amplicons from the first and second amplification rounds were firstly confirmed by restriction fragment length polymorphism (RFLP) with *Hae* III enzyme (Invitrogen™ Life Technologies, USA). The reaction was performed following the instructions of the manufacturer.

2.8. DNA sequencing

Amplicons from the first and second amplification round were purified by QIAquick PCR Purification Kit (Qiagen, USA) and sequenced in Mega Bace 1000/Automated 96 Capillary DNA Sequencer (Amersham Biosciences, UK). The quality of each sequence obtained was analyzed with Phred/Phrap/Consed (http://www.phrap.org/) software and the sequence similarity was checked against sequences deposited in the GenBank using the BLAST software.

2.9. Sensitivity

To assess the lowest detection limit of the SN-PCR the cell culture adapted BCoV Kakegawa strain (HA titre: 256) was 10-fold serially diluted in maintenance medium. RNA was extracted using the nucleic acid extraction method as mentioned earlier.

2.10. Specificity

For evaluation of the SN-PCR assay specificity faecal specimens were included containing enterotoxigenic *E. coli* ($n = 5$), bovine group A rotavirus ($n = 10$) and *Cryptosporidium* sp. ($n = 5$), detected respectively, by bacteriological routine tests, polyacrylamide gel electrophoresis technique and modified Ziehl-Nielsen method.

2.11. Statistical method

The kappa statistic (K) was calculated to evaluate the agreement between RT-PCR and SN-PCR systems. When $K = 0$ there is no agreement, $K < 0.3$ the agreement is poor, K between 0.3 and 0.5 is acceptable, K between 0.5 and 0.7 is good and $K > 0.7$ is excellent (Martin and Bonnet, 1987).

3. Results

Among the 50 diarrhoeic faecal samples included in this study the N gene of BCoV was detected in 4 (8%) samples by RT-PCR

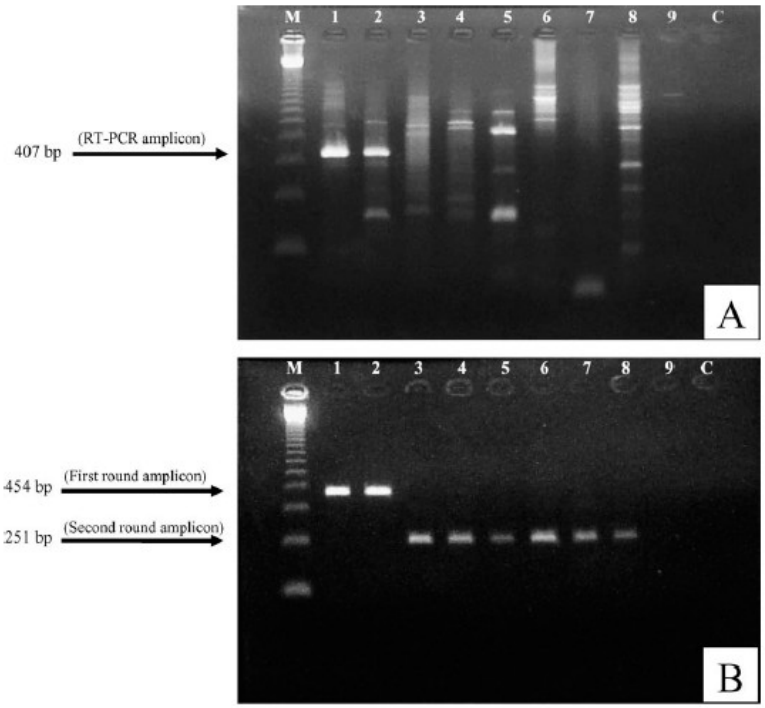

Fig. 1. Amplification of bovine coronavirus (BCoV) N gene in faecal samples from naturally infected calves by RT-PCR (panel A) and by SN-PCR (panel B). The BCoV specific amplicons are indicated by arrows in the left margin. Lane M: 123 bp ladder (Invitrogen™ Life Technologies, USA); lane 1: positive control (BCoV Kakegawa strain); lanes 2–9: faecal samples from diarrhoeic calves; lane C: negative control.

and in 12 (24%) samples by SN-PCR ($K = 0.43$). In fresh faecal samples ($n = 25$) the BCoV was detected in four samples by both, RT-PCR and SN-PCR in the first amplification round. No frozen faecal sample was BCoV positive by RT-PCR. However, in the SN-PCR developed in this study eight frozen faecal samples ($n = 25$) were BCoV positive in the second amplification round (Fig. 1). No positive results were obtained in normal faecal samples from asymptomatic calves by either of the PCR assays.

The specificity of SN-PCR amplicons from the first and second amplification rounds of the BCoV Kakegawa strain and the clinical samples were confirmed by RFLP with the *Hae* III enzyme. The amplicons of 454 bp (first round) and 251 bp (second round) from SN-PCR yielded fragments of, respectively, 366 and 88 bp, and 163 and 88 bp (Fig. 2). In addition, these amplicons were sequenced and submitted to BLAST analysis. Both techniques confirm that the 454 and 251 bp amplicons were BCoV specific.

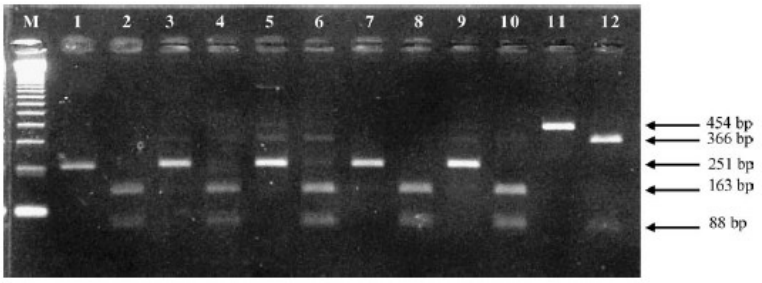

Fig. 2. Ethidium bromide stained agarose gel electrophoresis of restriction fragment length polymorphism (RFLP) with *Hae* III enzyme of BCoV N gene amplicons. Lane M: 123 bp ladder (Invitrogen™ Life Technologies, USA); lanes 1, 3, 5, 7 and 9: BCoV N gene SN-PCR amplicons; lanes 2, 4, 6, 8 and 10: RFLP of BCoV N gene SN-PCR amplicons digested with *Hae* III; lane 11: BCoV N gene first round amplicon; lane 12: RFLP of BCoV N gene first round amplicon digested with *Hae* III. Lanes 1–8, 11 and 12: diarrhoeic faeces from naturally infected calves; lanes 9 and 10: BCoV Kakegawa strain.

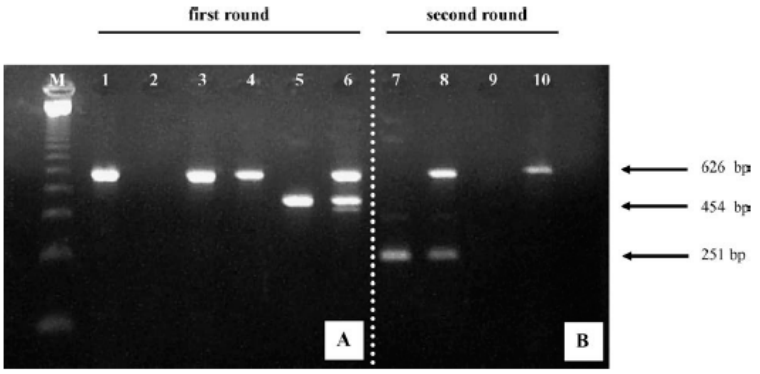

Fig. 3. Amplicon analysis by agarose gel electrophoresis of bovine coronavirus N gene (454 bp for first round and 251 bp for second round) and bovine mitochondrial ND5 gene (internal control, 626 bp) from bovine faecal samples analyzed by SN-PCR. Lane M: 123 bp ladder (Invitrogen™, Life Technologies, USA). Panel A: Faecal sample from asymptomatic (lanes 1–3, calf 55) and diarrhoeic (lanes 4–6, calf 17) calves. First round amplification with internal control primers (lanes 1 and 4) and BCoV specific primers (lanes 2 and 5) in single reaction, and in multiplex reaction (lanes 3 and 6). Panel B: Faecal sample from diarrhoeic (lanes 7 and 8, calf 15) and asymptomatic (lanes 9 and 10, calf 58) calves. Second round amplification in single reaction with BCoV specific primers (lanes 7 and 9) and in multiplex reaction with BCoV and internal control primers (lanes 8 and 10).

The extracted RNA of the BCoV Kakegawa strain (HA titre: 256) was amplified by SN-PCR until 10^{-7} dilution showing the sensitivity of the assay. No cross-reactivity was found when the SN-PCR was applied to faecal samples collected from calves with neonatal diarrhoea caused by enterotoxigenic *E. coli*, bovine group A rotavirus and *Cryptosporidium* sp. infections. No false positive or false negative results were observed in SN-PCR reactions with ultrapure sterile water or BCoV Kakegawa strain that were both included in all experiments.

The internal control primers (BOV1 and BOV2) used in a multiplex reaction in the first amplification round of the SN-PCR, or separately in two reactions, yielded a specific 626 bp amplicon in all diarrhoeic faecal samples analyzed in this study (Fig. 3).

4. Discussion

To improve the detection rate of BCoV in diarrhoeic fecal samples from naturally infected calves we developed a SN-PCR assay with an internal control and compared with a RT-PCR already described. The agreement between the two assays was only acceptable ($K = 0.43$). We worked with previously diluted samples subjected to long-term storage in freezer and with fresh faecal samples to verify the sensitivity assay under of the storage conditions. There was agreement between the two PCR methods in the detection rate of BCoV in fresh samples. However, the RT-PCR assay failed to amplify BCoV in frozen faeces. Of the 25 frozen samples the BCoV N gene could be detected in eight of them by the SN-PCR only. The employment of the second amplification round allowed a 200% increase in BCoV positive results in the samples included in this study.

We can exclude the possibility that our positive results in the frozen faecal samples were the result of intersample contamination. Careful measures were adopted to avoid the carry-over risk. Negative and positive controls accompanied the clinical samples in all the steps (nucleic acid extraction, RT, PCR and SN-PCR) where each step was carried-out in separate rooms. Besides, all the positive results were reanalysed (nucleic acid extraction until SN-PCR procedures) including the controls and the normal faeces in the same run.

A study by Van der Hoek et al. (1995), demonstrated that no appreciable loss in PCR signal was seen with long-term storage of faecal samples. They detected HIV RNA in faeces which had been stored for 9 years at −70 °C by RT-PCR. Therefore, in a retrospective study, SN-PCR developed in this study might be useful to detect BCoV in stored samples.

The three primers used in SN-PCR were designed from the published sequence of the N gene of the BCoV Mebus strain. Careful primer design can aid PCR optimization and improve greatly the assay (Butler et al., 2001). The SN-PCR primers possess very similar melting temperatures, with only two degrees of difference between them. Furthermore, only one primer-dimer formation was noted. Also of importance is the target region of BCoV RNA to be amplified. We chose the N gene because it is highly conserved among BCoV strains. It was concluded that the N protein is the most abundant antigen in coronavirus-infected cells because its RNA template is the smallest and it has the most abundant sgRNA (subgenomic RNA) during transcription (Hiscox et al., 2001). This indicates that there is more available RNA for the N gene than for the other BCoV protein genes. Consequently, detection of the N gene RNA might be advantageous due to its high abundance in cells, facilitating a high sensitivity of the diagnostic technique.

Tsunemtitsu et al. (1999) described the detection of BCoV RNA in faecal samples by RT-PCR and they also chose the N gene as target region for amplification. However, these authors worked with samples recently collected from experimentally inoculated adult cows while our BCoV positive results were obtained in faecal samples from calves with neonatal diarrhoea by natural infection. We tested the primers designed by Tsunemtitsu et al. (1999) (RT-PCR) to compare the efficiency of the amplification in frozen and fresh samples from naturally

infected calves. Although it was efficient for amplification of the positive control (Kakegawa strain), the same was not noted for clinical samples. The presence of unspecific amplicons and smears or failure to amplify the expected product disabled the diagnostic use. No absolute rules could be established to predict the ability of a primer pair to faithfully amplify a specific gene. However, the difference in detection rate may be the result of primer design. Analysis of the RT-PCR primers disclosed dimers and internal loop formation, palindrome sequences and different melting temperatures between the primers that can be related to the bad quality of the amplified products.

An adequate viral RNA extraction method is very important for molecular diagnosis. Four RNA extraction methods (TRIzol™, phenol/chloroform/isoamyl alcohol, guanidinium isothiocyanate, and a combination of phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate) were tested and compared for BCoV detection in faecal samples. SN-PCR detection rates after RNA extraction using the combination of phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate methods were better than after other RNA extraction protocols (data not shown). The combination of phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate methods was performed according to Barreiros et al. (2004) with slight modifications. Faecal samples remain the most difficult specimens for nucleic acid extraction and amplification due the presence of inhibitors (Monteiro et al., 1997). Guanidinium isothiocyanate alone or in combination with phenol–chloroform inactivates ribonucleases and therefore improves the stability of the extracted RNA genome (Romero, 1999). Van der Hoek et al. (1995), Hale et al. (1996), Rassol et al. (2002), Paula et al. (2003) also demonstrated that the guanidinium isothiocyanate method successfully removed inhibitors from faecal samples.

An internal control was included in the SN-PCR reactions to avoid false-negative results due to the presence of PCR inhibitors. This is particularly critical for faecal samples, in which the presence of inhibitors might affect the amplification process.

Epidemiological data on the frequency of BCoV occurring in South American livestock farms is very limited. In studies conducted in France, Spain, Costa Rica and Britain, BCoV was detect by antigen-capture ELISA in 6.8%, 7.3%, 9% and 14%, respectively (Reynolds et al., 1986; De la Fuente et al., 1998; Pérez et al., 1998; Naciri et al., 1999). However, the real frequency of the BCoV in neonatal calf diarrhoea outbreaks depends of the diagnostic technique. Cho et al. (2001) showed that nested PCR was 5000 times more sensitive than the antigen-capture ELISA. Therefore, the applicability of a sensitive technique such as PCR is fundamental to not underestimating the real prevalence of BCoV. Cho et al. (2001) also described a second round of amplification with the same primers described by Tsunemtitsu et al. (1999). However, this primer pair was tested only in fresh samples from experimentally infected calves and without an internal control.

We conclude that the SN-PCR described here is a useful tool for the diagnosis of BCoV in faeces from naturally infected calves. Differences in the ability to amplify and the characteristics of the PCR systems available for BCoV detection in clinical samples should be considered before use a test in future epidemiological studies. The rate of 24% positive diarrhoeic faecal samples confirms that the BCoV is an important etiological agent of neonatal diarrhoea in dairy and beef Brazilian cattle herds. The availability of a sensitive and specific diagnostic technique such as the SN-PCR described in this work will make it possible to undertake larger epidemiological studies of BCoV involved in the neonatal calf diarrhoea aetiology.

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4. ARTIGOS PARA PUBLICAÇÃO

4.1. MOLECULAR ANALYSIS OF BOVINE CORONAVIRUS S1 GENE BY DIRECT SEQUENCING OF DIARRHEIC FECAL SPECIMENS

Artigo editado de acordo com as normas de publicação do periódico ***Virus Research***

Molecular analysis of bovine coronavirus S1 gene by direct sequencing of diarrheic fecal specimens

Abstract

Bovine coronavirus (BCoV) causes severe diarrhea in newborn calves and is associated with winter dysentery in adult cattle and respiratory infections in calves and feedlot cattle. The BCoV S protein, plays a fundamental role in viral attachment and entry into the host cell and is cleaved into two subunits termed S1 (amino terminal region) and S2 (carboxy terminal region). This study describes a strategy for direct sequencing of the BCoV S1 subunit from diarrheic fecal samples and molecular analysis based on the genomic differences between Brazilian strains and other known BCoV from different geographical regions. To avoid the potential effects of cell culture-related nucleotide mutations, the nucleotide sequence without previous propagation in cell culture, that is an uncommonly performed procedure, was predicted. The phylogenetic analysis of the entire S1 nucleotide and deduced amino acid sequences showed that the BCoV Brazilian strains were more distant from the Mebus strain by 97.8% and 96.8% respectively, and more similar to the BCoV-ENT strain by 98.7% and 98.7% respectively. Based on the phylogenetic analysis of the hypervariable region of the S1 subunit these strains clustered with the American (BCoV-ENT, 182NS), Canadian calf diarrhea (BCQ20, BCQ7373, BCQ2070, BCQ9, BCQ571, BCQ1523) and the Canadian winter dysentery (BCQ2590) strain, but clustered on a separate branch of the Korean and respiratory BCoV strains. The BCoV strains of this study were not clustered in the same branch of previously published Brazilian strains (***AY606193***, ***AY606194***). These data corroborate with the genealogical construction and suggest the existence of at least two different BCoV strains circulating in Brazil.

Keywords: bovine coronavirus; S1 gene, sequencing, genetic polymorphism

1. Introduction

Bovine coronavirus (BCoV) is a member of the family *Coronaviridae*, order *Nidovirales* that causes severe diarrhea in neonatal calves, winter dysentery in adult cattle and has also been associated with respiratory infections in calves and feedlot cattle (Cho et al.,

2000; Clark, 1993). BCoV belongs to antigenic group 2 of the coronaviruses that includes murine hepatitis coronaviruses (MHV), porcine hemagglutinating encephalomyelitis virus (HEV), rat coronavirus (RtCoV) and human respiratory coronavirus (HCoV-OC43) (Lai and Holmes, 2001). Although the coronavirus is responsible for severe acute respiratory syndrome (SARS-CoV) is phylogenetically divergent from the three known antigenic groups of coronaviruses, the International Committee on the Taxonomy of Viruses has listed SARS-CoV in group 2. The virion, that possesses a single-stranded positive sense RNA genome of 32 kb length, encodes five major structural proteins: the nucleocapsid (N), the transmembrane (M), the hemaglutinin esterase (HE), the spike (S) and the small protein (E) (Lai and Cavanagh, 1997).

The S protein, a type I glycoprotein that forms the peplomers on the virion surface, binds to specific cellular receptors and is the major target of neutralizing antibodies (Cavanagh, 1995). Some group 2 coronaviruses, such as BCoV and HCoV-OC43, bind to 9-O acetylated sialic acid-containing receptors to initiate viral attachment (Krempl et al., 1995; Schultze and Herrler, 1992). The S protein is cleaved into two subunits (S1 and S2) by cellular trypsin-like proteases during processing in the Golgi complex. The amino-terminal S1 subunit, that forms the globular head of the mature protein, contains a receptor binding domain (RBD) as well as a hypervariable region (HVR). The carboxy terminal S2 subunit, a transmembrane protein, is required to mediate fusion of viral and cellular membranes (Gallagher and Buchmeier, 2001; Weiss and Navas-Martin, 2005).

Using probabilistic models, Wu and Yan (2005) propose that the S glycoprotein is more sensitive to mutations among coronavirus proteins from different species. Of the two cleavage products, the S2 subunit is highly conserved among bovine coronaviruses and natural genetic variability is more frequent in the S1 fragment. Mutations within both the RBD and HVR of S1 have been demonstrated to mediate coronavirus pathogenesis (Weiss

and Navas-Martin, 2005). Because of this, most molecular studies relative to genetic diversity among coronaviruses are performed with the S1 protein.

Like most RNA viruses, coronaviruses are believed to mutate at a high frequency due to the lack of proffreading activity in their RNA polymerases. Based on an estimation of one error per 10,000 bases, the coronavirus RNA polymerase may generate approximately three random mutations in each 30 kb genome produced in an infected cell (Malpica et al., 2002). This fact should be evaluated when clinical specimens are adapted and isolated in cell culture before the sequencing procedure. BCoV propagation in cell culture may also affect the antigenic composition (Hussain et al., 1991), hemagglutination activity (Storz et al., 1992), and intestinal replication of the virus (Kapil et al., 1990). Fundamental changes of amino acids (aa) composition of the S protein have been described into the JHM strain of MHV after extensive propagation in tissue culture (Gallagher and Buchmeier, 2001). Mutations in the membrane protein of the SARS-CoV were already observed in passage 0 (Poon et al., 2005). Thus, it is very important to consider such events when interpreting data during molecular analysis. However, there are few comparative studies relative to S protein mutations in BCoV isolates in cell lines and direct sequencing from clinical specimens are not commonly performed. To the author's knowledge, BCoV S1 complete sequences representative of the South America wild type strains have not been published. This study describes a strategy for the direct sequencing of the S1 gene of BCoV from fecal samples and molecular analysis based on the genomic differences between Brazilian strains and others BCoV from different geographical regions.

2. Materials and methods

2.1. Virus and cells

The HRT-18 cells were grown in Dulbecco's Modified Eagle's Medium (D-MEM, Gibco BRL, USA), supplemented with 10% fetal bovine serum (FBS, Gibco BRL, USA), 55 μg/ml gentamicine (Sigma Co., USA) and 2.5 μg/ml amphotericin B (Sigma Co., USA). The Kakegawa strain of BCoV was propagated in HRT-18 cells cultured in FBS free D-MEM and used as positive control in the RT-PCR assay.

2.2. Clinical specimens

Three BCoV positive fecal samples (BR-UEL1, BR-UEL2 and BR-UEL3) were obtained during the winter of 2004 from calves up to 30 days old with clinical signs of diarrhea from a Brazilian dairy cattle herd, Minas Gerais State (21^{o} 41' 49" S; 45^{o} 18' 45" W). The fecal samples were diluted 2-fold in 0.01 M phosphate-buffered saline (PBS) pH 7.2 (137 mM NaCl; 3 mM KCl; 8 mM Na_2HPO_4; 15 mM KH_2HPO_4) and centrifuged at 3000 x *g* for 15 min at 4 ^{o}C. The supernatants were used for RNA extraction. The samples were previously identified as BCoV by RT-PCR for N gene detection (Takiuchi et al., 2006).

2.3. RNA extraction

Aliquots of 400 μl from fecal suspensions were treated with SDS at a final concentration of 1% (w/v), homogenized by vortexing and kept at 56 ^{o}C for 30 min. For RNA extraction, a combination of phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate methods was performed according to Barreiros et al. (2004) with slight modifications. Briefly, 400 μl of phenol/chloroform/isoamyl alcohol (25:24:1) were added, vortexed and heated at 56 ^{o}C for 15 min (Sambrook et al., 1989). The mixture was then centrifuged at 10,000 g for 10 min and the supernatant was transferred into a new tube and processed by the silica/guanidinium isothiocyanate method (Boom et al., 1990). The RNA was eluted from the silica pellet with 50 μl of diethyl-pyrocarbonate (DEPC) treated sterile

water by 15 min incubation at 56 ^{o}C and centrifugation at 10,000 g for 10 min. The supernatant fraction was kept at –20 ^{o}C until use.

2.4. Primers

Seven pairs of primers were designed based on the S gene sequence of BCoV Mebus strain and were located in regions where eight other known BCoV strains (BCoV-ENT, LSU-94, OK-514-3, LY-138, Quebec, F15 and the avirulents BCoV Vaccine and L9) exhibit conserved nucleotide (nt) sequences. For the sequences alignment and design of the primers were used the following softwares: CLUSTAL W Multiple Alignment Program (http://www.ebi.ac.uk/clustalw/) and Gene Runner version 3.05 (Hastings Software Inc., Hastings, NY) (http://www.generunner.com), respectively. The S1 gene of BCoV was reverse transcribed and PCR amplified in seven overlapping fragments. Details of the primers used for RT-PCR and sequencing strategy of the S1 gene are indicated in Table 1 and Fig. 1, respectively.

2.5. RT-PCR

The reverse transcription (RT) reaction was performed with 8 μl of extracted RNA and 2 μl of the random primer pdN6 (GE Healthcare, Little Chalfont, UK) that was incubated at 97 ^{o}C for 4 min. The mixture was placed on ice for 5 min and 10 μl of RT mix containing 1x RT buffer (50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 75 mM KCl), 0.1 mM of each dNTP (Invitrogen^{TM} Life Technologies, USA), 10 mM DTT, 100 units of M-MLV Reverse Transcriptase (Invitrogen^{TM} Life Technologies, USA) and ultrapure sterile water (MilliQ) to a final volume of 20 μl. The mixture was incubated at 37 ^{o}C for 60 min and followed by inactivation at 95 ^{o}C for 5 min.

For amplification, 8 μl of the RT reaction were added to 42 μl of the PCR mix consisting of 1.5 x PCR buffer (30mM Tris-HCl pH 8.4 and 75mM KCl), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μl (20 pmol) of each primer (Table 1), 2.5 units *Platinum Taq* DNA polymerase (Invitrogen™ Life Technologies, USA) and ultrapure sterile water to a final volume of 50 μl. The reaction was performed in a thermocycler PTC-200 (MJ Research Co. Water Town, MA, USA) with the following time and temperature conditions: one step of 4 min/94 ^{o}C; followed by 40 cycles of 1 min/94 ^{o}C, 1min/52 ^{o}C, 1 min/72 ^{o}C and a final step of 7 min/72 ^{o}C.

The products were analyzed by electrophoresis in a 2% agarose gel in TBE buffer pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA), stained by ethidium bromide (0.5 μg/ml) and visualized under UV light.

2.6. Nucleotide sequencing and alignment

The PCR amplicons were purified using GFX PCR DNA and Gel Band Purification (GE Healthcare, Little Chalfont, UK) and sequenced in MegaBACE 1000/Automated 96 Capillary DNA Sequencer (GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. Sequencing was performed in both directions using the forward (F) and reverse (R) primers corresponding to each PCR amplicon. The quality of each sequence obtained was analyzed with Phred/Phrap/Consed Analysis Program (http://www.phrap.org) and the sequence identity was verified with sequences deposited in the GenBank using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST). Firstly, the BCoV nt sequences from this study were aligned and compared with the full-length S1 sequence of other known BCoV strains (Table 2) by the CLUSTAL/W method. In the second analysis, based on the HVR region of the S1 gene, we also included other BCoV strains from

distinct geographical regions. The degree of similarity among sequences at both nt and aa levels was determined using the software BIOEDIT v. 5.0.9 (Hall, 1999).

2.7. Phylogenetic analysis

The obtained alignments were used as input for phylogenetic analysis using the software Mega version 3.1 (Kumar et al., 2004). For the dendrogram construction only strains with published full-length S sequences (Mebus, BCoV-ENT, LSU, OK, LY-138, F15, Vaccine and L9) were included in the analysis. The root was inferred with the corresponding torovirus (B145 strain) homologous sequence as outgroup. Phylogenetic trees of S1 gene sequences were constructed using heuristic search and equal weighting in the maximum-parsimony (MP) analysis replications. For the construction of MP unrooted tree based on the nt sequence of the BCoV HVR (1,368 to 1,776) were included in this analysis other published BCoV sequences from different countries (Table 2).

2.8. Nucleotide sequence accession number

The descriptions and nt GenBank accession numbers of BCoV strains used in this study are summarized in Table 2.

3. Results

A consensus sequence of 2681 nt, that spans nt 25 to 2705 of the BCoV Mebus strain, was obtained through direct sequencing of the seven overlapping PCR fragments of the S gene from three wild-type BCoV detected in diarrheic calves. Fig. 2 shows the seven overlapping PCR fragments obtained from the diarrheic fecal sample BR-UEL1. Specific amplicons with strong intensity and absence of unspecific bands and smears indicate the

excellent quality of the amplified products. Likewise, this quality standard was obtained with the BR-UEL2, BR-UEL3 and Kakegawa strains.

Except for the Kakegawa strain, the BCoV BR-UEL samples did not undergo cell culture passage prior to PCR amplification and sequencing. In this study, the nt identity between the BR-UEL strains was 100% and gaps, insertions or deletions were not observed after the sequences alignment.

Over the nt sites a total of 58 nucleotides substitutions were identified within BCoV BR-UEL wild-type strains in comparison to the BCoV prototype Mebus strain, of which 33 (56.9%) were transitions and 25 (43.1%) transversions. However, there were only 5 nt positions that differentiated the Kakegawa strain from the ancestral Mebus strain. In comparison to the deduced S protein of the Mebus strain, these substitutions led to 28 and 5 aa changes in the Brazilian wild-type strains and Kakegawa strain respectively. We identified 3 aa mutations that were exclusive to BCoV BR-UEL strains including aa positions 141 (Q→L), 510 (S→I) and 767 (R→S). Data from the comparison of the predicted aa sequences of the BCoV BR-UEL and Kakegawa strains, with the other known BCoV are summarized in Table 3.

To examine the relationships between BCoV BR-UEL and other BCoV strains within the S gene, the nt and aa identity percentages among BCoV sequences were calculated. In both nt and aa analysis, the BR-UEL strains showed the lowest percentage of identity with the Mebus strain by 97.8% and 96.8% respectively, and the highest degree of sequence identity with the BCoV-ENT strain by 98.7% and 98.7% respectively.

Based on the total number of nt substitutions, a phylogenetic analysis was performed to create a rooted tree of the relationships among different BCoV strains. In this first analysis we included only BCoV strains with published full-length S1 sequences (Mebus, BCoV-ENT, LSU-94, OK-514-3, LY-138, F15, Vaccine, L9) and the appropriate bredavirus sequence was

included as outgroup rooting. Phylogenetic tree was constructed using the maximum parsimony algorithms with reliability estimated in 1000 bootstrap replications. Phylogenetic data showed that BCoV BR-UEL strains clustered with the enteric BCoV-ENT and the respiratory strains LSU-94 and OK-514-3. The Kakegawa strain clustered in a separate group with Mebus, Quebec and BCoV vaccine strains (Fig.3)

The region spanning nt residues 1368 to 1776 (aa 452 to 593) of the BCoV S1 gene has been identified as a hypervarible region (Rekik and Dea, 1994). In this study we also performed the phylogenetic analysis based on the HVR from BCoV BR-UEL strains and other BCoV known published sequences. Besides the 10 reference strains that were analyzed previously we included other 19 relevant BCoV strains that GenBank entries had not available the S1 full sequence. We attempted to include in this analysis isolates from a variety of geographical regions to verify the relationship between them. The unrooted tree using maximum parsimony based on the S1 HVR nt sequences revealed that BCoV BR-UEL wild-type strains clustered in groups with the American strains (BCoV-ENT, 182NS), Canadian BCQ calf diarrhea strains (BCQ20, BCQ7373, BCQ2070, BCQ9, BCQ571, BCQ1523) and an Canadian BCQ Winter Dysentery strain (BCQ2590). The second cluster included Korean calf diarrhea (KCD) and WD strains (KWD), American and Canadian respiratory strains (OK-514-3, LSU-94, 220NS, 232NS, BCO44175, BCO43277, BCQ3994). The other Brazilian strains (USP3, USP4) and Mebus, Kakegawa, Quebec, vaccine, F15, LY-138 reference strains were clustered on a separate branch (Fig.4).

4. Discussion

The BCoV S protein, a glycoprotein of 4092 nt long, is responsible for viral attachment and entry into the host cell. Variations in host range and tissue tropism is largely attributable to mutations in the S glycoprotein of coronaviruses. The S glycoprotein is cleaved

into two subunits termed S1 (amino terminal region) and S2 (Carboxy terminal region). Phylogenetic studies have been demonstrated that the S1 subunit, a glycoprotein of 2,304 nt long, is more sensitive to genetic variability than S2 subunit (Gallagher and Buchmeier, 2001).

In this study we design a strategy for the sequencing of the BCoV S1 gene directly from fecal diarrheic specimens that were previously identified as BCoV positive by RT-PCR assay for N gene detection (Takiuchi et al., 2006). Over the 4092 nt that compose the S gene, we successfully identified 2681 nt that spans nt 25 to 2705 of the prototype BCoV Mebus strain.

These isolates did not undergo cell culture passage prior to sequencing, except for the Kakegawa strain that was used as positive control in our procedures. The Kakegawa strain was originally isolated from fecal sample of an adult cow with winter dysentery in Japan (Akashi et al., 1980). To the best of the author's knowledge, this is the first submission of S1 full-sequence of Kakegawa strain in public databases.

Early studies have already described the S1 gene partial amplification and direct sequencing from fecal diarrheic samples without previous inoculation in culture cells (Brandão et al., 2006; Liu et al., 2006). However, this is the first report of the entire S1 gene amplification directly from fecal samples by RT-PCR and subsequent sequence analyses. The nt sequences obtained by direct sequencing from clinical specimens is more reliable since we have eliminated the potential effect of cell culture-related nt mutations. These mutations may occur during viral adaptation to cell culture, because the molecular clock accelerates during periods of environmental changes. Tong et al. (2004) described evidences for the existence of nt mutations in SARS-CoV associated with cell-culture adaptation. The same observation was reported in canine coronavirus obtained from fecal samples in comparison to canine coronavirus reference strains grown in cell cultures (Naylor et al., 2001). Although studies

performed by Hasoksuz et al. (2002) and Jeong et al. (2005) have already described the complete sequence of the BCoV S1 gene, all strains were previously propagated in HRT-18 cells before sequencing. Altlhough the feces are considered as the most difficult clinical sample for nucleic acid extraction and amplification due to the presence of PCR inhibitors (Monteiro et al., 1997), we successfully obtained the complete S1 sequence without passage in culture cell.

In the present study, the phylogenetic analysis was performed in two steps. Initially, the complete nt sequences of the S1 gene from the three BR-UEL wild-type strains, the Kakegawa strain and other 10 BCoV published sequences (Table 2) were aligned and analyzed. In this analysis we intended to locate the BR-UEL strains and to compare to the others known BCoV strains since that this is the first description of the S1 full sequence in BCoV strains from the South America. In addition, although only the S1 partial sequence of Kakegawa strain has been submitted to the GenBank (accession number AY646095), phylogenetic study that included it has not been reported so far.

The sequence alignments demonstrated that BR-UEL strains were 100% identical. By paired comparisons the BR-UEL strains were closely related to the enteric BCoV-ENT strain, reaching an identity of 98.7% by nt and aa analysis. In contrast, BR-UEL strains were genetically more distant from the Mebus strain (97.8% and 96.8%) in nt and aa analysis, respectively. Other studies also described similar phylogenetic difference between field isolates and the ancestral enteric Mebus strain (Hasoksuz et al., 2002; Jeong et al., 2005; Liu et al., 2006). However, the Kakegawa and Mebus strains demonstrated the highest level of nt (99.8%) and aa (99.4%) identity. These data are in accordance with Fukutomi et al. (1999) who described the close relationship between Mebus and Kakegawa strains by cross virus neutralization tests.

The rooted tree generated after nt alignment emphasizes the relationship among the isolates analyzed. The BR-UEL wild-type strains were located in the same cluster of the BCoV-ENT strain and in a segregated cluster of the Mebus strain. On the other hand, the Kakegawa and Mebus strains were in the same cluster. The value of 1000 bootstrap replications and the inclusion of a bredavirus as outgroup support this phylogenetic inference (Fig.3).

In the second step of the phylogenetic study we also included other relevant BCoV strains from different geographical regions (North and South Americas, Asia, Europe) with S1 partial sequences available in the GeneBank database (Table 2). We selected the HVR of the BCoV S1 gene, that spanning nt 1368 to 1776 (aa 452 to 593), as target domain for the molecular analysis. The HVR contains the antigenic domain II (aa 517 to 621) of the S protein, which is neutralized by monoclonal antibodies. Yoo and Deregt (2001) have described that a single amino acid change within antigenic domain II was responsible for the escape of BCoV from virus neutralization. Similarly, MHV also contains an immunodominat $CD8^+$ T-cell epitope within HVR and aa changes within this domain have been reported as a mechanism to escape the immune response and induce viral persistence (Weiss and Navas-Martin, 2005).

Chouljenko et al. (1998) has proposed that aa changes at positions 510, 531, 543 and 578 may be specific for respiratory strains. However, aa change at position 543 of respiratory strains (LSU and OK) was identical in the BR-UEL enteric strains (Table 3). Similarly, Hasoksouz et al. (2002) and Jeong et al. (2005) have also identified identical substitutions as described by Chouljenko et al. (1998) at aa 510, 543 and 578 positions in their enteric and winter dysentery BCoV strains. Further, at aa 531, the enteric BCQ 1523 strain also has the same substitution as described in the BCoV respiratory strains (Jeong et al., 2005). Therefore,

these data do not support those described by Chouljenko et al. (1998), since the same changes were observed in BCoV strains with varied tissue tropism.

In some coronaviruses, the S1 subunit also contains a receptor binding domain (RBD) which plays a role in the interaction between spike and the host cell. The RBD for BCoV is currently unknown. However for the MHV S protein, the RBD has been localized at the amino terminal 330 amino acids of S1 (Kubo et al., 1994) and have been described that a single amino acid substitution within the RBD affects the virulence of the strain. By BLAST database analysis a 55% identity between MHV and BR-UEL coronaviruses strains in the corresponding region was identified. Based on this domain, we detected 15 aa substitutions in the BR-UEL strains when compared to the Mebus strain (Table 3). Jeong et al. (2005) also described 15 aa substitutions in the Korean winter dysentery strain and suggest that it may induce either an alteration of the receptor-binding ability during viral invasion or pathogenicity to cattle. However these substitutions identified in the Korean strain were located in different sites of the BCoV BR-UEL strains.

The maximum-parsimony unrooted tree based on HVR nt sequences suggest the existence of three distinct clusters. The BR-UEL wild-type strains clustered with BCoV-ENT strain and other Canadian calf diarrhea and winter dysentery strains. Interestingly, BR-UEL strains clustered in a separate branch of the other strains circulating in Brazil (USP strains) (Fig.4). Brandão et al. (2006) reported the first description of a gap of 18 nt (deletion of 6 aa) within HVR in their Brazilian BCoV isolates. Deduced aa comparisons revealed 8 aa substitutions between the Brazilian strains (BR-UEL and USP). Similarly BR-UEL and Mebus strains showed 10 aa changes in this genomic segment (data not shown). Therefore, these data corroborate with the genealogical construction and suggest the existence of at least two different BCoV strains circulating in Brazil.

BCoV infections often result in high morbidity but usually in low mortality. Interestingly, BR-UEL wild-type strains belong to a sampling obtained during an outbreak of neonatal calf diarrhea that culminated with the death of calves in a Brazilian dairy cattle herd. Three aa changes at positions 141 (Q→L), 510 (S→I) and 767 (R→S) were exclusive in BR-UEL strains and have not yet been observed in any previously published sequences when compared to the Mebus strain. Since important domains, such as RBD for BCoV, has yet to be elucidated, additional experimentation will be fundamental to predict whether these aa changes have potential effects on the pathogenesis of BCoV.

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Table 1. Oligonucleotide primers designed from the spike gene of the BCoV for RT-PCR and sequence analysis.

Primer pair		Sequence (5' to 3')	Position	Polarity
A	SPK1_F	ATG TTT TTG ATA CTT TTA ATT	1-21	Positive
	SPK1_R	ATT GGT AGT ATG TGG TGT	420-438	Negative
B	SPK2_F	TAT GGC ACT GAA GGG AAC	231-248	Positive
	SPK2_R	CTA TTA CAA GTC AAA GGC A	743-761	Negative
C	SPK3_F	TGG CAT TGG GAT ACA GGT	550-567	Positive
	SPK3_R	CAA GTA AAT GAG TCT GCC T	1103-1121	Negative
D	SPK4_F	GCA GAT GTT TAC CGA CGT	955-972	Positive
	SPK4_R	TAC ACA CAA AGA CCC ATC C	1473-1491	Negative
E	SPK5_F	GAA TTG ATA CTA CTG CTA C	1256-1274	Positive
	SPK5_R	TCA TAA TTA ACA CAA ACA CC	1885-1904	Negative
F	SPK6_F	GTA ATC CTT GTA CTT GCC	1721-1738	Positive
	SPK6_R	TAG TAA ACC GAT AAC CAG T	2314-2332	Negative
G	SPK7_F	TAA CTC TTC CGA ACC AGC A	2085-2103	Positive
	SPK7_R	AAT CGC TTC CTA AAC AAC C	2701-2719	Negative

Table 2. Bovine coronavirus (BCoV) strains, origin country and GenBank accession number used in maximum parsimony analysis of spike glycoprotein sequences.

Strain	Country	GenBank accession
BR-UEL1 [a, b]	Brazil	***DQ479421***
BR-UEL2 [a, b]	Brazil	***DQ479422***
BR-UEL3 [a, b]	Brazil	***DQ479423***
Kakegawa [a, b]	Japan	***DQ479424***
Mebus [a, b]	USA	***U00735***
LY-138 [a, b]	USA	***AF058942***
BcoV-ENT [a, b]	USA	***AF391541***
LSU-94 [a, b]	USA	***AF058943***
OK-514-3 [a, b]	USA	***AF058944***
Vaccine [a, b]	USA	***M64668***
L9 [a, b]	USA	***M64667***
F15 [a, b]	France	***D00731***
Québec [a, b]	Canada	***AF220295***
BCQ7373 [b]	Canada	***AF239306***
BCQ1523 [b]	Canada	***AF239307***
BCQ2590 [b]	Canada	***AF239317***
BCQ3994 [b]	Canada	***AF339836***
BCO44175 [b]	Canada	***AF239309***
BCO43277 [b]	Canada	***AF239308***
BCQ571 [b]	Canada	***AH010363***
BCQ9 [b]	Canada	***U06091***
BCQ20 [b]	Canada	***U06092***
BCQ2070 [b]	Canada	***U06090***
KCD2 [b]	South Korea	***DQ389633***
KCD4 [b]	South Korea	***DQ389635***
KCD10 [b]	South Korea	***DQ389641***
KWD7 [b]	South Korea	***AY935643***
KWD8 [b]	South Korea	***AY935644***
182 NS [b]	USA	***DQ320764***
232 NS [b]	USA	***DQ320763***
220 NS [b]	USA	***DQ320762***
USP3 [b]	Brazil	***AY606193***
USP4 [b]	Brazil	***AY606194***
Bredavirus B145	Netherlands	***AJ575373***

a: S1 full sequence phylogenetic analysis

b: S1 hypervariable region phylogenetic analysis

Table 3. Polymorphic sites between amino acid residues 9 to 900 in the spike gene from BCoV BR-UEL wild-type strains and others BCoV reference strains. Dashes represent consensus with the Mebus strain.

	Amino acid position																	
Strain	**11**	**13**	**33**	**36**	**40**	**88**	**100**	**115**	**118**	**141**	**146**	**148**	**154**	**169**	**173**	**175**	**179**	**181**
Mebus	M	F	A	I	I	R	I	K	M	Q	N	D	L	H	H	N	K	V
BR-UEL1	T		V		T	T	T	D	K	L	–	–	F	N	N	–	Q	–
BR-UEL2	T	–	V	–	T	T	T	D	K	L	–	–	F	N	N	–	Q	–
BR-UEL3	T	–	V	–	T	T	T	D	K	L	–	–	F	N	N	–	Q	–
Kakegawa	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	K	–	–
BCoV_ENT	T	–	V	–	T	T	T	D	–	–	I	G	F	N	N	–	Q	–
LSU94	T	–	V	–	T	T	T	D	K	–	–	–	–	N	N	–	Q	–
OK514	T	–	V	–	T	T	T	D	K	–	–	–	–	N	N	–	Q	–
LY138	–	L	V	V	T	T	–	N	–	–	–	–	–	–	–	–	R	I
Quebec	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	K	–	–
F15	–	L	V	–	T	T	–	H	–	–	–	–	–	N	–	–	R	–
Vaccine	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	Q	–
L9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Strain	**248**	**253**	**256**	**362**	**370**	**378**	**425**	**440**	**447**	**458**	**465**	**470**	**483**	**484**	**499**	**501**	**509**	**510**
Mebus	L	S	M	S	D	D	T	V	T	F	V	H	P	S	N	P	N	S
BR-UEL1	M	N	L	–	–	–	–	–	–	S	A	D	–	T	S	S	–	I
BR-UEL2	M	N	L	–	–	–	–	–	–	S	A	D	–	T	S	S	–	I
BR-UEL3	M	N	L	–	–	–	–	–	–	S	A	D	–	T	S	S	–	I
Kakegawa	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–
BCoV_ENT	M	N	L	–	Y	–	–	–	–	S	A	D	S	T	–	–	T	–
LSU94	M	N	L	–	–	–	–	–	–	S	A	D	–	T	S	S	–	T
OK514	M	N	–	–	–	–	–	–	I	S	A	D	–	T	S	S	–	T
LY138	M	N	–	–	–	–	–	–	–	S	–	D	–	T	S	S	–	–
Quebec	–	–	–	–	–	–	–	–	–	–	–	–		K	–	–	–	–
F15	M	N	–	–	–	–	–	L	I	S	–	D	–	T	–	–	–	–
Vaccine	–	Y	–	–	–	E	A	–	–	S	–	–	–	T	–	–	–	–
L9	–	N	–	C	–	–	–	–	–	S	–	–	–	T	–	–	–	–

Strain	**520**	**531**	**543**	**571**	**578**	**580**	**606**	**648**	**651**	**707**	**742**	**744**	**767**	**769**	**778**	**817**	**834**
Mebus	N	N	S	Y	T	Q	L	V	T	I	S	V	R	A	T	T	Y
BR-UEL1	–	D	A	H	–	–	–	–	–	–	–	–	S	S	N	–	–
BR-UEL2	–	D	A	H	–	–	–	–	–	–	–	–	S	S	N	–	–
BR-UEL3	–	D	A	H	–	–	–	–	–	–	–	–	S	S	N	–	–
Kakegawa	K	D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BCoV_ENT	–	D	A	H	–	–	–	–	–	–	–	–	–	S	N	–	–
LSU94	–	G	A	–	S	–	–	–	–	F	–	–	–	S	N	–	–
OK514	–	G	A	–	S	R	–	–	–	–	–	–	–	S	N	–	–
LY138	–	–	–	–	–	–	–	–	–	–	–	A	–	–	N	I	–
Quebec	–	D	–	–	–	–	F	–	–	–	–	–	–	–	N	–	–
F15	–	–	–	–	–	–	–	A	–	–	A	A	V	–	N	–	C
Vaccine	–	D	–	–	–	–	–	–	–	–	–	–	–	–	N	–	–
L9	–	D	–	–	–	–	–	–	P	–	–	–	–	–	N	–	–

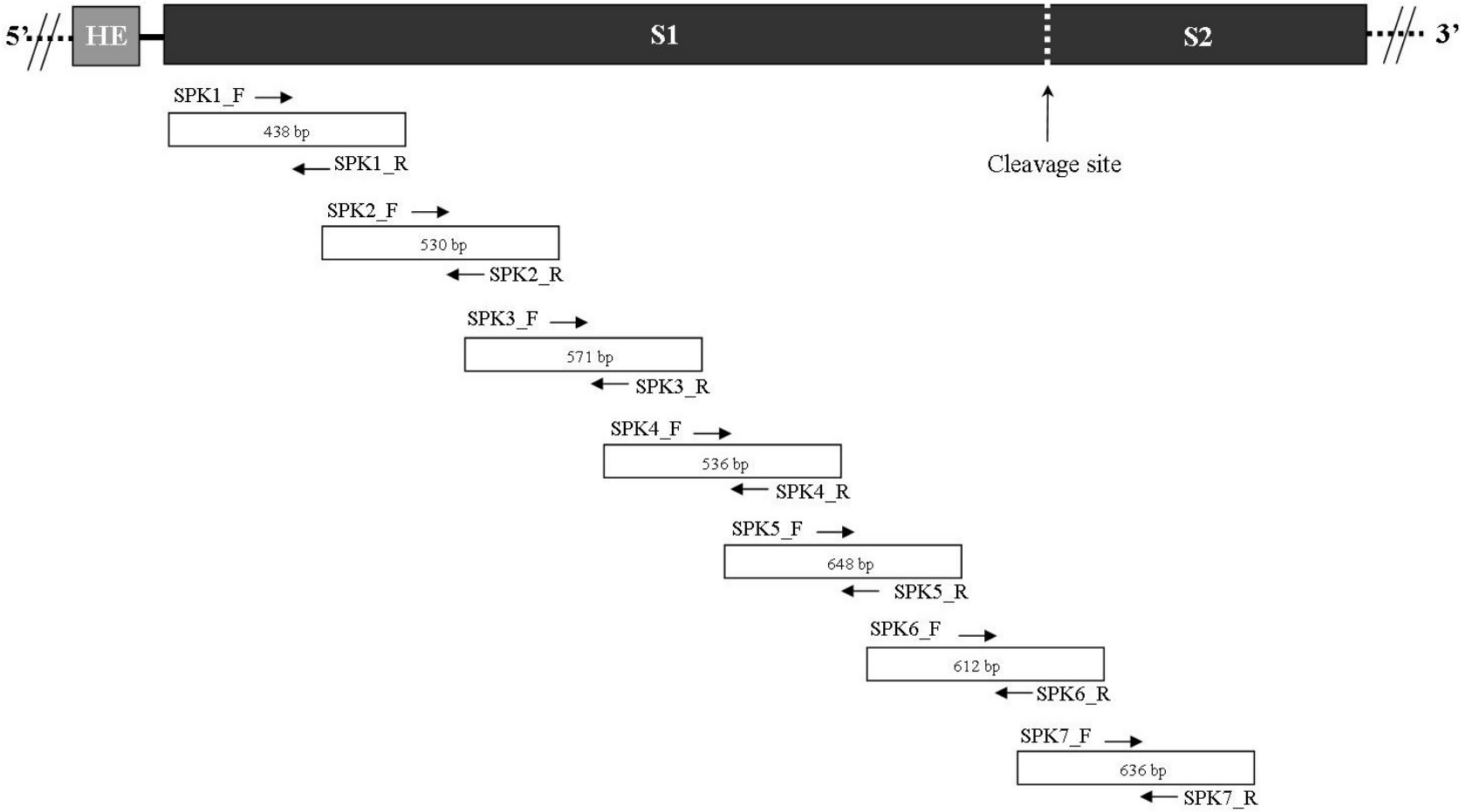

Fig.1. Schematic diagram of the BCoV spike gene and sequencing strategy. The expected product length for each overlapping PCR fragment is indicated into the white box. The oligonucleotide primers used for RT-PCR and DNA sequencing are shown as arrows pointing towards the direction of DNA synthesis.

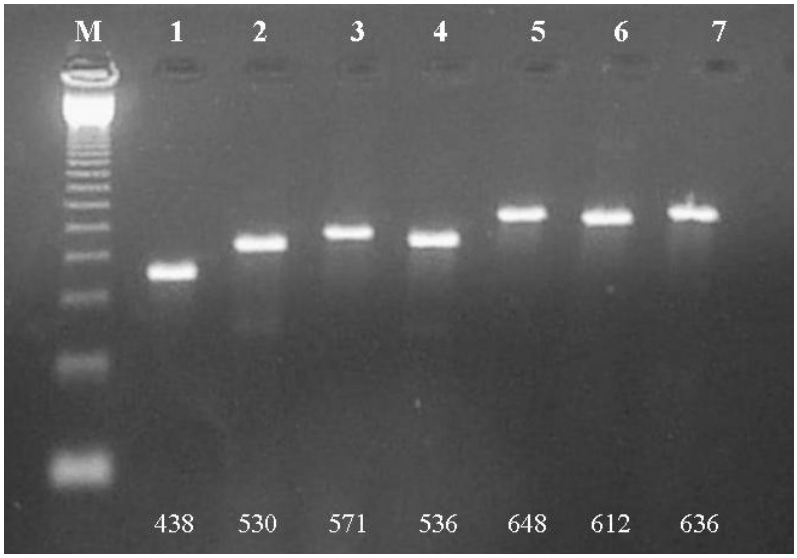

Fig. 2. RT-PCR of spike gene of the BR-UEL1 wild-type bovine coronavirus strains. M: ladder, 123 bp (Invitrogen™ Life Technologies, USA); lanes 1 to 7: Seven overlapping PCR products of the S gene amplified with the primers pairs SPK1 to SPK7.

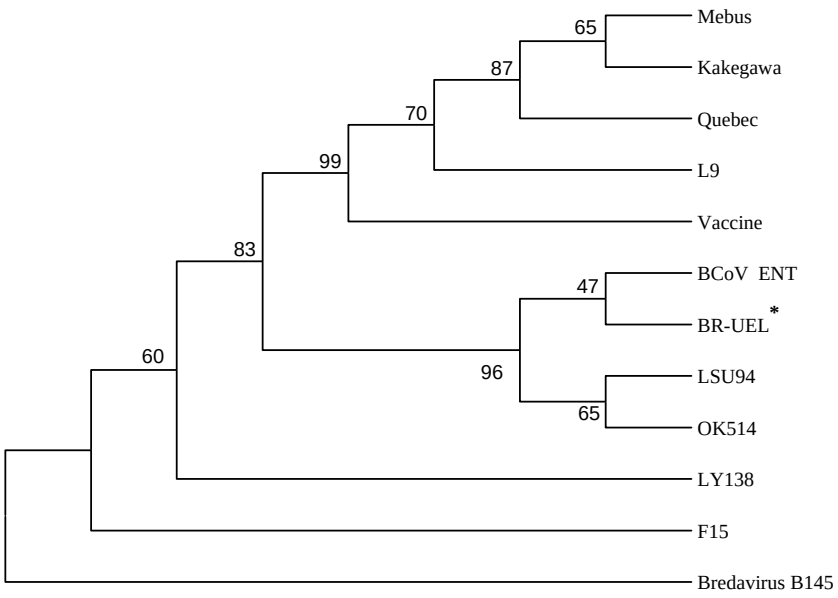

Fig. 3. Dendrogram of the maximum-parsimony rooted tree based on the nucleotide sequence of S1 gene of BCoV strains. A bredavirus strain was used as outgroup. Bootstrap values are shown in the main branch nodes. The asterisk marks identical sequences (BR-UEL1, BR-UEL2, BR-UEL3).

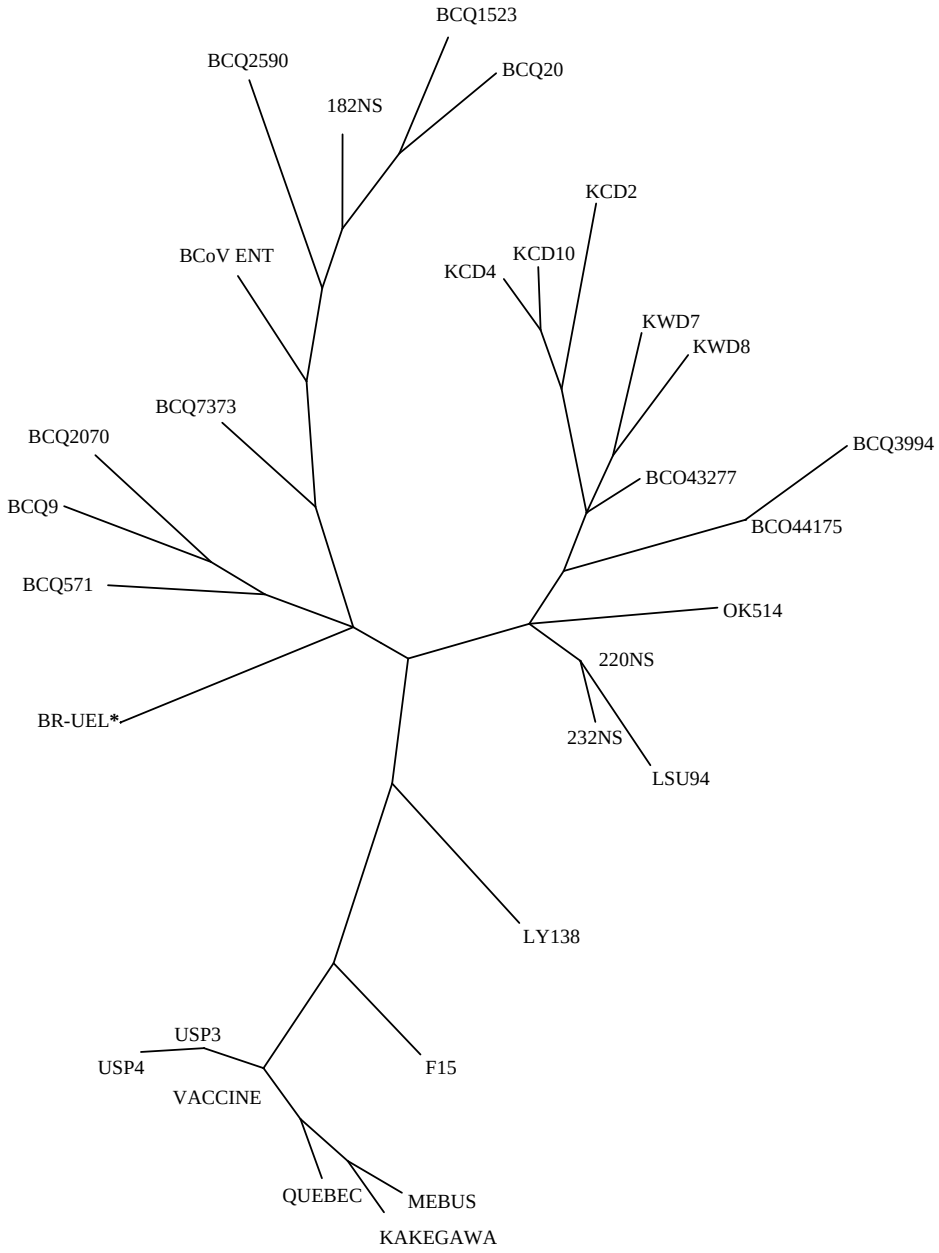

Fig.4. Maximum-parsimony unrooted tree based on the nucleotide sequence of the hypervariable region of BCoV strains. The asterisk marks a group of identical nucleotide sequences (BR-UEL1, BR-UEL2, BR-UEL3).

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4.2. IDENTIFICATION OF A MUTATION IN THE SPIKE PROTEIN CLEAVAGE SITE IN BRAZILIAN STRAINS OF WILD-TYPE BOVINE CORONAVIRUS

Artigo editado de acordo com as normas de publicação do periódico ***Archives of Virology***

Identification of a mutation in the spike protein cleavage site in Brazilian strains of wild-type bovine coronavirus

Summary

The spike (S) protein of coronaviruses is a type I membrane glycoprotein that is primarily responsible for entry into susceptible cells by binding with specific receptors on cells and mediating subsequent virus-cell fusion. The bovine coronavirus (BCoV) S protein is cleaved into two subunits, the N-terminal S1 and the C-terminal S2. The proteolytic cleavage site of S protein is highly conserved among BCoV strains and is represented by the basic amino acids sequence KRRSRR. This study described a single mutation in the S protein cleavage site of three Brazilian strains of BCoV detected in diarrheic samples from calves naturally infected. The PCR sequenced products revealed that amino acid sequence of the cleavage site of our strains was KRRSSR, indicating an amino acid mutation at position 767 (R→ S). This amino acid substitution occurred due only a single nucleotide substitution in the proteolytic cleavage site, CGT to AGT. This is the first description of this nucleotide mutation (C to A), that resulted in the change of the codon for arginine to serine. In this study we speculated the probable effects of this mutation in the proteolytic cleavage site using the murine hepatitis coronavirus as a comparative model.

Brief Report

Bovine coronavirus (BCoV), a member of the family *Coronaviridae*, order *Nidovirales*, belongs to group 2 of the coronaviruses which include murine hepatitis coronaviruses (MHV), porcine hemagglutinating encephalomyelitis virus (HEV), equine coronavirus (ECoV), rat coronavirus (RtCoV) and human respiratory coronavirus (HCoV-OC43) [8]. BCoV is an enveloped virus with single-stranded, positive-sense RNA genome of approximately 32 kb length that encodes five major structural proteins: the nucleocapsid (N),

the transmembrane (M), the hemaglutinin esterase (HE), the spike (S) and the small protein (E) [12]. The S glycoprotein of BCoV is a large membrane glycoprotein of approximately 150 kDa that forms the characteristic peplomers (club-shaped structures) on the virion surface. The S protein is primarily responsible for the entry of coronavirus into susceptible cells by binding to specific receptors on cells and mediating virus-cell fusion and subsequent cell-cell fusion during infection [4].

In several coronavirus, such as infectious bronchitis virus (IBV), MHV, BCoV, as a late event in maturation, the protein is cleaved into two subunits: S1 (aminoterminal region) and S2 (carboxyterminal region) [16]. Proteolytic cleavage of these coronaviruses spike protein occurs adjacent to a sequence of basic amino acids on the carboxyterminal region of S1. In the S protein of BCoV, the predicted basic amino acid sequence that is involved in cleavage by the host cell-derived proteolytic enzyme is KRRSRR. This sequence, highly conserved between BCoV strains, encompasses amino acids 763 to 768; cleavage occurs between amino acids 768 and 769 [18]. The cleavage of the S protein has been reported as a process related to the viral infectivity and cell fusion in many group 2 coronaviruses. Studies related to MHV, the best-studied member of the *Coronavirus* family, demonstrated that cleavage of S is not essential for infectivity but is associated with enhanced cell fusion (syncytia) in infected cell monolayers [6, 7, 19].

This study describes a single mutation in the S protein cleavage site of wild type Brazilian strains of BCoV observed in calves naturally infected and speculates the possible effects of this mutation at the proteolytic cleavage site using the MHV as a comparative model.

Three BCoV positive fecal samples (BR-UEL1, BR-UEL2 and BR-UEL3) were obtained from calves up to 30 days old with clinical signs of diarrhea in a Brazilian dairy herd from Minas Gerais State (21^{o} 41' 49" S; 45^{o} 18' 45" W). Fecal samples were diluted 2-fold in

0.01 M phosphate-buffered saline (PBS) pH 7.2 (137 mM NaCl; 3 mM KCl; 8 mM Na_2HPO_4; 15 Mm KH_2HPO_4), centrifuged at 3000 x *g* for 15 min at 4 ^oC to remove the supernatants were used for RNA extraction. Aliquots of 400 µl from fecal suspensions were treated with SDS at a final concentration of 1% (w/v), homogenized by vortexing and kept at 56 ^oC for 30 min.

For RNA extraction a combination of phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate methods was performed according to Barreiros et al. [1] with slight modifications. Briefly, 400 µl of phenol/chloroform/isoamyl alcohol (25:24:1) were added, vortexed and heated at 56 ^oC for 15 min [15]. The mixture was centrifuged at 10,000 g for 10 min and the supernatant was transferred into a new tube and processed by the silica/guanidinium isothiocyanate method [2]. The RNA was eluted from the silica pellet with 50 µl of diethyl-pyrocarbonate (DEPC) treated sterile water by 15 min incubation at 56 ^oC and centrifugation at 10,000 g for 10 min. The supernatant fraction was kept at –20 ^oC until use.

Specific oligonucleotide primers that flank the sequence corresponding to S protein cleavage site were designed using the Gene Runner program version 3.05 (Hastings Software Inc., Hastings, NY) (http://www.generunner.com). The primers sequences (positions calculated from the start codon of the S gene) were: SPK7_F: 5'-TAACTCTTCCGAACCAGCA-3' (nt 2085-2103) and SPK7_R: 5'-AATCGCTTCCTAAACAACC-3' (nt 2701-2719), amplifies a predicted fragment of 636 bp.

The reverse transcription (RT) reaction was performed with a mixture of 8 µl of extracted RNA and 2 µl of the random primer pdN6 (GE Healthcare, Little Chalfont, UK) and was incubated at 97 ^oC for 4 min. Subsequently, it was placed on ice for 5 min and 10 µl of RT mix containing 1x RT buffer (50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 75 mM KCl), 0.1 mM of each dNTP (Invitrogen^{TM} Life Technologies, USA), 10 mM DTT, 100 units of M-

MLV Reverse Transcriptase (Invitrogen™ Life Technologies, USA) and ultrapure sterile water to a final volume of 20 μl were added and incubated at 37 °C for 60 min and followed by inactivation at 95 °C for 5 min.

For amplification, 8 μl of the RT reaction were added to 42 μl of the PCR mix consisting of 1.5 x PCR buffer (30 mM Tris-HCl pH 8.4 and 75m M KCl), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μl (20 pmol) of each primer, 2.5 units *Platinum Taq* DNA polymerase (Invitrogen™ Life Technologies, USA) and ultrapure sterile water to a final volume of 50 μl. The reaction was performed in a thermocycler (PTC-200, MJ Research Co. Water Town, MA, USA) with the following time and temperature conditions: one step of 4 min/94 °C; followed by 40 cycles of 1 min/94 °C, 1min/52 °C, 1 min/72 °C and a final step of 7 min/72 °C.

The products were analyzed by electrophoresis in a 2% agarose gel in TBE buffer pH8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA), stained with ethidium bromide (0.5 μg/ml) and visualized under UV light.

The PCR amplicons were purified using GFX PCR DNA and Gel Band Purification (GE Healthcare, Little Chalfont, UK) and sequenced in MegaBACE 1000/Automated 96 Capillary DNA Sequencer (GE Healthcare), according to the manufacturer's instructions. Sequencing was performed in both directions using the forward (F) and reverse (R) primers corresponding to each PCR amplicon. The quality of each sequence obtained was analyzed with Phred/Phrap/Consed Analysis Program (http://www.phrap.org) and the sequence identity verified with sequences deposited in the GenBank using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequences of the wild-type Brazilian BCoV strains were aligned and compared with the BCoV reference strains using the CLUSTAL/W program.

The sequences reported in this study have been deposited in the GenBank database under accession numbers: DQ479421 (BR-UEL1), DQ479422 (BR-UEL2) and DQ479423

(BR-UEL3). The predicted secondary structure, hydrophilicity profile and protease map was determined by the program Protean of DNAStar software (DNAStar, Inc., Madison, WI, USA).

The sequenced PCR products revealed that amino acid sequence of the cleavage site of the three wild-type Brazilian BCoV strains was KRRSSR, indicating an amino acid mutation at position 767 (R→ S). The unique mutational description in the proteolytic cleavage in bovine coronaviruses was reported in 1991 with the wild type French BCoV strain F-15, where the amino acid substitution also occurred at position 767 (R→V) (Fig 1). However, the authors did not discuss the effects related to the mutant amino acid [22].

The BCoV BR-UEL sequences are genuine and not the result of RT-PCR or sequencing errors, since the same mutation was successful amplified and sequenced in all three samples and in different fecal aliquots from the same animal. Besides, the potential effects of cell culture-related nucleotide mutations were eliminated, since the samples did not undergo cell culture passage prior to PCR amplification and sequencing.

Although there are no study related to mutational analysis of the proteolytic cleavage site in BCoV strains, this phenomenon has been previously described in MHV strain. Computational analyses demonstrated that the HCoV-OC43, MHV-A59 and BCoV present high degree of identity among group 2 coronaviruses [17]. Based on these evidences, the MHV may be used as an appropriate model to hypothesize the effects of the new mutation observed in the S protein cleavage signal of wild-type BCoV strains during this study.

Cleavage of S protein is related to efficient cell-cell fusion (syncytia) by MHV in infected cell monolayers but is not necessary for virus-cell fusion (infectivity). The MHV spike proteins have mutations that eliminate cleavage into S1 and S2 subunits carry out cell-to-cell fusion very inefficiently; however, they mediate entry into susceptible cells with similar efficiency as wild-type virus [3, 7, 13]. Although the cleavage of the S protein is not a

prerequisite for fusion, it does enhance the induction of syncytia [3]. Hingley et al. [11] and Hann et al. [10] related that the cleavage of the S protein of MHV-A59 mutant was greatly reduced compared to the wild-type virus. The sequence at the predicted cleavage site of MHV-A59 wild type is RRAHR while that of MHV-A59 mutant is RRADR. The MHV-A59 mutant substituted a negatively charged aspartic acid (D) for the weakly basic histidine (H) in the cleavage signal [7]. These authors suggest that the introduction of a negatively charged amino acid into this highly basic region destroys the signal or otherwise prevents its usage and thereby inhibits cleavage of the spike.

Similar to MHV, BCoV S gene also has a proteolytic cleavage site formed by a group of basic amino acids upstream from the S2 amino terminal region. Except for BCoV F15, this sequence is highly conserved in all known BCoV strains (KRRSRR). In the BCoV BR-UEL samples we identified an amino acid mutation (R→S) at aa position 767 (KRRSSR) (Fig.1). This amino acid substitution occurred due only to a single nucleotide mutation at the proteolytic cleavage site, CGT to AGT. This mutation (C to A), that resulted in the change of the codon for arginine to serine has never been described. The cleavage into the two subunits occurs between amino acid 768 and 769 and is thought to be mediated by cellular trypsin-like proteases [18]. The protease map indicated that three BR-UEL strains lack one predicted cleavage site by trypsin when compared with other known BCoV. In MHV studies there are strong evidence that furin enzyme is the cell protease responsible for cleavage of the S protein in cultured cells [9]. In addition, although did not alter the predicted secondary structure or hydrophilicity of the protein, the substitution of arginine to serine is a nonconservative substitution. Nonconservative amino acid changes within a single protein may result in alterations of the physical energy of the protein-protein interaction and may destabilize the protein native conformation [5]. Navas-Martin et al. [14] suggest that the proper spatial arrangement of the S1 and S2 subunits is crucial for the biological functions of the S protein.

Although a single aa mutation seems to be inconsistent, Yoo and Deregt [21] generated BCoV mutants and confirmed that a single point mutation at domain II of S1 protein was responsible for the escape of BCoV from immunological response. Similarly, He et al. [10] described that a single amino acid substitution in the receptor-binding domain of SARS coronavirus S protein disrupted the antigenic structure and binding activity. In addition, a single amino acid change has already been demonstrated to influence MHV ability to spread within the central nervous system [20].

Recently, Navas-Martin et al. [14] have demonstrated that a single cleavage signal substitution in MHV mutants may play a major role in virulence. These authors have associated this mutation with higher viral load and highly virulent phenotypes. Interestingly, the BR-UEL were obtained during an outbreak of neonatal diarrhea that culminated with the death of calves in a Brazilian dairy cattle herd. Because BCoV infections resulting in high mortality is uncommon, the results described by Navas-Martin et al. [14] for MHV strain should be also investigated for BCoV proteolytic cleavage site mutants.

We are uncertain if this single mutation prevented the normal proteolytic cleavage of the S protein or increased viral load, as occurred in MHV mutants. Therefore, additional experiments must be done to determine the effects of the amino acid change (R→S) within the proteolytic cleavage site on the structural and functional characteristics of the BCoV S protein. Development of a system which introduces infectious cDNA clones with specific mutations into the BCoV genome will provide an important tool to determine the role of these BCoV mutants in the pathogenesis of the neonatal calf diarrhea.

Acknowledgments

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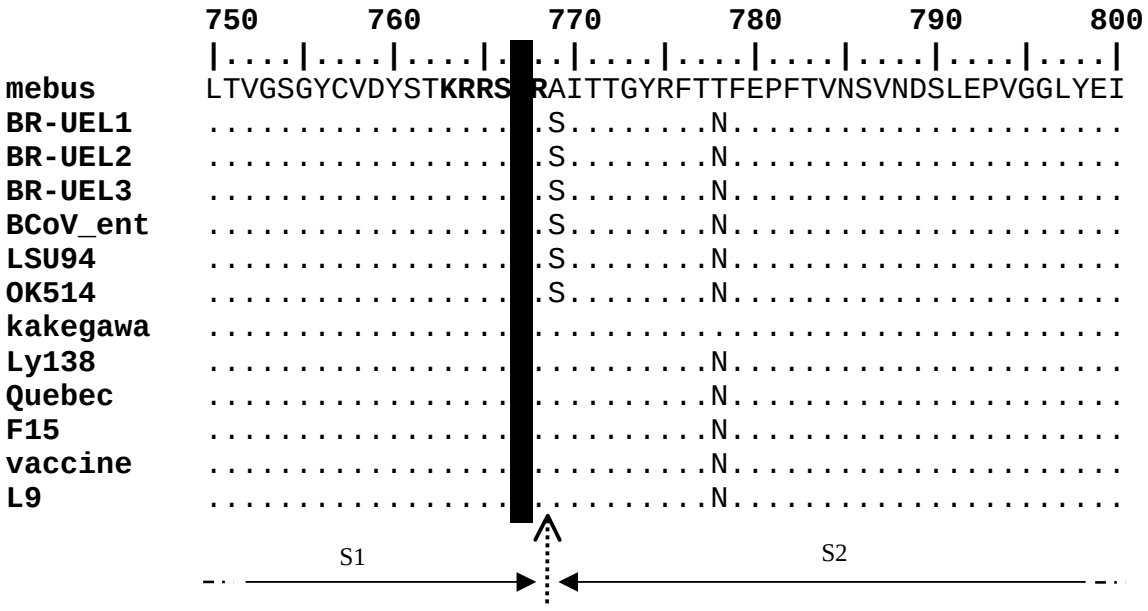

Fig. 1. Predicted amino acid sequences for the spike gene in the cleavage site proximities. Residues in bold represents proteolytic cleavage signal sequence of the Mebus strain. The arrow between amino acids 768 and 769 indicates the cleavage site between S1 and S2. Shaded residue indicates the mutation in the cleavage signal. BCoV BR-UEL strains (GenBank accession numbers DQ479421, DQ479422, DQ479423), Mebus (U00735), BCoV ENT (AF391541), LSU94 (AF058943), OK514 (AF058944), Kakegawa (DQ479424), LY138 (AF058942), Quebec (AF220295), F15 (D00731), Vaccine (M64668), L9 (M6466).

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5. CONCLUSÕES

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- A técnica de semi-nested PCR desenvolvida para o diagnóstico do BCoV pela amplificação parcial do gene N apresentou alta sensibilidade e especificidade. A inclusão de um controle interno para a detecção do fragmento do DNA mitocondrial bovino em sistema multiplex-PCR possibilitou maior precisão no diagnóstico do BCoV;

- O emprego da SN-PCR proporcionou incremento nos resultados positivos para a detecção do BCoV, quando comparado com a utilização da RT-PCR descrita na literatura;

- A estratégia de seqüênciamento por sobreposição do gene S1 do BCoV foi satisfatória para a obtenção da seqüência consenso em material fecal sem a prévia propagação do vírus em cultivo celular. A análise filogenética das estirpes de BCoV indicou a existência de três grupos distintos e fica proposta a ocorrência de pelo menos duas estirpes diferentes de BCoV circulantes nos rebanhos bovino brasileiros;

- As estirpes brasileiras do BCoV descritas neste estudo contém uma mutação inédita (R→S) na seqüência de aminoácidos correspondente ao sítio de clivagem da proteína S.

APÊNDICES

APÊNDICE A: Lista de Reagentes

1. 100 mM dNTP Set, 4 x 250 µl; 25µmol each (100 mM dATP Solution, 100 mM dCTP Solution, 100 mM dGTP Solution, 100 mM dTTP Solution) (Invitrogen Life Technologies®)
2. 10 x PCR-Buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen Life Technologies®)
3. 123 bp DNA Ladder (Invitrogen Life Technologies®)
4. 2-Mercapto-ethanol ($C_2H_6O_5$) P.M. 78,13 (Fluka®)
5. 5 x RT-Buffer (250 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 375 mM KCl) (Invitrogen Life Technologies®)
6. Acetona, P.A. (CH_3COCH_3) P.M. 58,08 (Dinâmica®)
7. Ácido acético glacial, P.A. (CH_3COOH) P.M. 60,05 (Nuclear®)
8. Ácido bórico (H_3BO_3) P.M. 61,83
9. Ácido clorídrico (HCl) P.M. 36,46 (Reagen®)
10. Ácido etilenodiaminotetraácido Sal di-sódico – EDTA, P.A. ($C_{10}H_{14}N_2O_8Na_22H_2O$) P.M. 372,24 (Reagen®)
11. Agarose (Gibco BRL®)
12. Álcool etílico absoluto (C_2H_2OH) P.M. 46,07 (Nuclear®)
13. Álcool isoamílico ($(CH_3)_2CHCH_2CH_2OH$) P.M. 88,15 (Synth®)
14. Azul de bromofenol (Sigma®)
15. Cloreto de Potássio, P.A. (KCl) P.M. 74,56 (Reagen®)
16. Cloreto de Sódio, P.A. (NaCl) P.M. 58,45 (Reagen®)
17. Clorofórmio, P.A. ($CHCl_3$) P.M. 119,38 (Dinâmica®)
18. Didhiothreitol – DTT (Invitrogen Life Technologies®)
19. Dodecil Sulfato de Sódio – Lauril Sulfato de Sódio – SDS ($C_{12}H_{25}NaO_4S$) P.M. 288,38 (Synth®)
20. Ethidium bromide ($C_{21}H_{20}N_3Br$) P.M. 394,3 (Sigma®)
21. Glicina, P.A. (Nuclear®)
22. Guanidine isothiocyanate P.M. 118,16 (Gibco BRL®)
23. *Hae* III (5'- GG↓CC -3', 3'- CC↑GG – 5') 2500 units; 10 U/µl (Invitrogen Life Technologies®)
24. Hidróxido de Sódio, P.A. (NaOH) P.M. 40,00 (Dinâmica®)

25. Hidroximetil amino metano – TRIS 99% P.M. 121,14 (Inlab®)
26. Metanol, P.A. (CH_3OH) P.M. 32,04 (Allkimia®)
27. M-MLV Reverse Transcriptase - 200 units/µl (Invitrogen Life Technologies®)
28. PdN(6) Random Hexamer (GE Healthcare®)
29. *Platinum* Taq DNA Polymerase recombinant 500 units (Invitrogen Life Technologies®)
30. REACT® 2 (500 mM Tris-HCl, 100 mM $MgCl_2$, 500 mM NaCl) (Invitrogen Life Technologies®)
31. Sacarose, P.A. – sucrose ($C_{12}H_{22}O_{11}$) p.m. 342,31 (Reagen®)
32. Silicon dioxide (SiO_2) P.M. 60,08 (Sigma®)
33. Triton x-100

APÊNDICE B: Soluções e Tampões

- **Hidratação da sílica**
  - 60 g de sílica (SIGMA®)
  - Adicionar 500 mL de água MilliQ autoclavada
  - Agitar lentamente e manter em repouso durante 24 horas
  - Por sucção, desprezar 430 mL do sobrenadante
  - Ressuspender a sílica em 500 ml de água bidestilada
  - Manter em repouso durante 5 horas para sedimentar
  - Desprezar 440 mL do sobrenadante
  - Adicionar 600 μL de HCl (32% w/v) para ajustar o pH (pH=2,0)
  - Aliquotar e autoclavar

- **Solução L6**
  - 120 g de tiocianato de guanidina (GUSCN)
  - 100 mL de TRIS-HCl 0,1 M pH 6,4
  - 22 mL de EDTA 0,2 M pH 8,0
  - 2,6 mL de Triton x 100

- **Solução L2**
  - 120 g de tiocianato de guanidina (GUSCN)
  - 100 mL de TRIS-HCl 0,1 M pH 6,4

- **Tampão de Amostra**
  - Azul de bromofenol 0,25%
  - Sacarose – sucrose ($C_{12}H_{22}O_{11}$) 45%

- **Tampão de corrida: TBE (TRIS – Ácido bórico – EDTA) 10 x [ ]**
  - 0,89 M TRIS
  - 0,89 M ácido bórico
  - 0,02 M EDTA dissodium
  - Água bidestilada qsp. 1 litro

    pH = 8,4

- **Tampão estabilizador de rotavírus (TRIS/Ca) 10 x**

  - 12,12g TRIS (0,89mM)
  - 2,2 g cloreto de cálcio (1,5mM)
  - Água MilliQ autoclavada q.s.p. 1 litro

  pH = 7,4

- **Tampão fosfato salina (PBS)**

  -137 mM cloreto de sódio (NaCl)
  - 3 mM cloreto de potássio (KCl)
  - 8 mM sódio fosfato dibásico anidro (Na_2HPO_4)
  - 15 mM potássio fosfato monobásico ($K_2H_2PO_{4)}$
  - Água MilliQ autoclavada q.s.p. 1 litro

  pH = 7,2

- **Fenol / clorofórmio – álcool isoamílico (24:24:1)**

  - 24 mL fenol saturado
  - 24 mL clorofórmio
  - 1 mL álcool isoamílico

APÊNDICE C: Protocolo de Técnicas

- **Suspensão fecal – Extração bruta**

  - Pesar 1g do extrato fecal em balança de precisão
  - Adicionar 9 mL de tampão TRIS/Ca 1X

    (Para amostras muito líquidas estabelecer proporção 1:2)
  - Homogeneizar
  - Calibrar tubos
  - Centrifugar 10 minutos a 3000 x g
  - Recolher sobrenadante
  - Identificar e estocar em frascos a 4º C

- **Extração do RNA: Método associação das técnicas fenol/clorofórmio – álcool isoamílico e sílica/ tiocianato de guanidina**

  - 400 μL da suspensão fecal
  - 400 μL de fenol / clorofórmio – álcool isoamílico
  - Homogeneizar em vórtex
  - Banho-maria à 56º C durante 15 minutos
  - Centrifugar a 10.000 x g durante 10 minutos
  - Recolher sobrenadante em outro tubo
  - 30 μL de sílica hidratada
  - 900 μL de solução L6
  - Homogeneizar em vórtex
  - Agitar durante 30 minutos em temperatura ambiente
  - Centrifugar a 10.000 x g durante 30 segundos
  - Desprezar sobrenadante em solução contendo NaOH 10M
  - Adicionar 500 μL de solução L2
  - Homogeneizar em vórtex
  - Centrifugar a 10.000 x g durante 30 segundos
  - Desprezar sobrenadante em solução contendo NaOH 10M
  - Adicionar 500 μL de solução L2
  - Homogeneizar em vórtex
  - Centrifugar a 10.000 x g durante 30 segundos

- Desprezar sobrenadante em solução contendo NaOH 10M
- Adicionar 1 mL de etanol a 70%
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g durante 30 segundos
- Desprezar sobrenadante
- Adicionar 1 mL de etanol a 70%
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g durante 30 segundos
- Desprezar sobrenadante
- Adicionar 1 mL de acetona PA
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g durante 1 minuto
- Desprezar sobrenadante
- Secar o pellet em banho-maria à 56^{o} C durante 15 minutos (tubo aberto)
- Adicionar 50 µL de água MilliQ autoclavada
- Homogeneizar em vórtex
- Banho-maria à 56^{o} C durante 15 minutos (tubo fechado)
- Homogeneizar em vórtex
- Centrifugar a 10.000 x g durante 2 minutos
- Recolher sobrenadante em eppendorf de 0,5 µL
- Estacar a -20ºC

- **Digestão enzimática dos produtos de PCR – *Hae* III**
  - 1 µL da enzima de restrição *Hae* III
  - 2 µL do tampão da enzima (REACT 2)
  - 3 µL de água ultrapura estéril
  - 15 µL do produto da PCR
  - Incubar a 37ºC durante 1 a 2 horas

- **Gel de agarose a 2%**
  - 1 g de agarose
  - 50 mL TEB 1 x
  - 30 µL ethidium bromide

ANEXOS

ANEXO A: Normas de Publicação do periódico *Virus Research*

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Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the Authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the Author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name, and, if available, the e-mail address of each Author.

Corresponding Author
Clearly indicate who is willing to handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address.**

Present/permanent address.
If an Author has moved since the work described in the article was done, or was visiting at the time, a "Present address"' (or "Permanent address") may be indicated as a footnote to that Author's name. The address at which the Author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract.
A concise and factual abstract is required (maximum length 200 words). The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separate from the article, so it must be able to stand alone.
References should therefore be avoided, but if essential, they must be cited in full, without reference to the reference list.
Non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords.
Immediately after the abstract, provide a maximum of 6 keywords,

avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations.
Define abbreviations that are not standard in this field at their first occurrence in the article: in the abstract but also in the main text after it. Ensure consistency of abbreviations throughout the article.

Arrangement of the article

Subdivision of the article.
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2,), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to "the text". Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction.
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Experimental/Materials and methods.
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results.
Results should be clear and concise.

Discussion.
This should explore the significance of the results of the work, not repeat them.

Conclusions.
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Acknowledgements.
Place acknowledgements, including information on grants received, before the references, in a separate section, and not as a footnote on the title page.

References.
See separate section, below.

Figure captions, tables, figures, schemes.
Present these, in this order, at the end of the article. They are described in more detail below. High-resolution graphics files must always be provided separate from the main text file (see Preparation of illustrations).

Footnotes.
Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves on a separate sheet at the end of the article. Do not include footnotes in the Reference list.

Table footnotes.
Indicate each footnote in a table with a superscript lowercase letter.

Tables.
Number tables consecutively with Arabic numerals in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. All tables must have descriptive headings and possible legends below; both headings and legends should be understandable without reference to the text. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

Nomenclature and units.
Follow internationally accepted rules and conventions: use the international system of units (SI). If other quantities are mentioned, give their equivalent in SI.

Virus nomenclature
Each virus should be identified at least once, preferably in the 'Introduction' or 'Materials and Methods' section, using formal family, genus, and species terms and where possible by using a precise strain designation term as developed by an internationally recognized specialty group or culture collection. Please note that the word type is not used before species designations that include a number. Formal terms used for virus families, genera, and species should be those approved by the International Committee on Taxonomy of Viruses (ICTV):Fauquet, C.M., Mayo, M.A.,Maniloff, J., Desselberger, U., and Ball, L.A.(2005) Virus Taxonomy, Classification and Nomenclature of Viruses. Eighth ICTV Report, Academic Press, an imprint of Elsevier. This volume also includes standard abbreviations for species. Once formal taxonomic names have been given in a paper, vernacular terms may be used.

Formal taxonomic nomenclature
In formal taxonomic usage, the first letters of virus order, family, subfamily, genus and species names are capitalized and the terms are printed in italics. Other words in the species names are not capitalized unless they are proper nouns or parts of nouns, for example *West Nile virus*. In formal usage, the name of the taxon should precede the term for the taxonomic unit; for example; "the family *Paramyxoviridae*," "the genus *Morbillivirus*." The following represent examples of full formal taxonomic terminology:
1. Order *Mononegavirales*, family *Rhabdoviridae*, genus *Lyssavirus*, species *Rabies virus*.
2. Family *Poxviridae*, subfamily *Chordopoxvirinae*, genus *Orthopoxvirus*, species *Vaccinia virus*.
3. Family *Picornaviridae*, genus *Enterovirus*, species *Poliovirus*.
4. Family *Bunyaviridae*, genus *Tospovirus*, species *Tomato spotted wilt virus*.

Vernacular Taxonmic Nomenclature
In formal vernacular usage, virus order, family, subfamily, genus and species names are written in lower case Roman script: they are not capitalized, nor are they printed in italics or underlined. In informal usage, the name of the taxon should not include the formal suffix, and the name of the taxon should follow the term for the taxonomic unit; for example "the picornavirus family, the enterovirus genus." One particular source of ambiguity in vernacular nomenclature lies in the common use of the same root terms in formal family, genus or species names. Imprecision stems from not being able to easily identify in vernacular usage which hierarchical level is being cited. For example, the vernacular name "*paramyxovirus*" might refer to the family *Paramyxoviridae*, or one species in the genus *Respirovirus*, such as *Human parainfluenza virus 1*. The solution in vernacular usage is to avoid "jumping" hierarchical levels and to add taxon identification wherever needed. For exam ple, when citing the taxonomic placement of *Human parainfluenza virus 1*, taxon identification should always be added: *Human Parainfluenza virus 1* is a

species in the genus *Respirovirus*, family *Paramyxoviridae*. In this example, as is usually the case, adding the information that this virus is also a member of the subfamily *Paramyxovirinae*and the order *Mononegavirales* is unnecessary.
It should be stressed that italics and capital initial letters need be used only if the species name refers to the taxonomic category. When the name refers to viral objects such as virions present in a preparation or seen in an electron micrograph, italics and capital initial letters are not needed and the names are written in lower case Roman script. This also applies when the names are used in adjectival form, for instance tobacco mosaic virus polymerase. The use of italics when referring to the name of a species as a taxonomic entity signals that it has the status of an officially recognized species. The 8th ICTV Report (Fauquet, C.M. et al., 2005, Academic Press, an imprint of Elsevier) should be consulted to ascertain which names have been approved as official species names. When the taxonomic status of a new putative species is uncertain or its position within an established genus has not been clarified, it is considered a tentative species and its name is not written in italics although its initial letter is capitalized.
Origins of bioreagents - The origins of bioreagents should be described adequately, including citation of culture collections, companies, or colleagues from whom the bioreagents were obtained. If viruses were collected from nature, the collecting site and procedure should also be properly described. Bioreagents include but are not necessarily limited to virus strains and species, antibodies, and cell lines.

GenBank/DNA sequence linking
Many Elsevier journals cite "gene accession numbers" in their running text and footnotes. Gene accession numbers refer to genes or DNA sequences about which further information can be found in the databases at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine. Elsevier Authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources, should type this information in the following manner:
For each and every accession number cited in an article, Authors should type the accession number in **bold, underlined text.** Letters in the accession number should always be capitalised. (See Example below). This combination of letters and format will enable Elsevier's typesetters to recognise the relevant texts as accession numbers and add the required link to GenBank's sequences.
Example: "GenBank accession nos. **AI631510** , **AI631511** , **AI632198** , and **BF223228)** , a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. **BE675048)** , and a T-cell lymphoma (GenBank accession no. **AA361117)** ".
Authors are encouraged to check accession numbers used very carefully. **An error in a letter or number can result in a dead link.** In the final version of the ***printed* article**, the accession number text will not appear bold or underlined. In the final version of the ***electronic* copy**, the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article.

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As a minimum, the full URL should be given. Any further information, if known (Author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.
Text:
All citations in the text should refer to:
1. *Single Author:* the Author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two Authors:* both Authors' names and the year of publication;
3. *Three or more Authors:* first Author's name followed by "et al." and the year of publication.
Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.
Examples: "as demonstrated (Allan, 1996a, 1996b, 1999; Allan and Jones, 1995). Kramer et al. (2000) have recently shown ...."
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same Author(s) in the same year must be identified by the letters "a", "b", "c", etc., placed after the year of publication.
Examples:
Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2000. The art of writing a scientific article. J. Sci. Commun. 163, 51-59.
Reference to a book:
Strunk Jr., W., White, E.B., 1979. The Elements of Style, third ed. Macmillan, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 1999. How to prepare an electronic version of your article, in: Jones, B.S., Smith, R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281-304.
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List of serial title word abbreviations: http://www.issn.org/lstwa.html
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The digital object identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. The correct format for citing a DOI is shown as follows (example taken from a document in the journal *Physics Letters B*):
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• Make sure you use uniform lettering and sizing of your original artwork.
• Save text in illustrations as "graphics" or enclose the font.
• Only use the following fonts in your illustrations: Arial, Courier, Helvetica, Times, Symbol.
• Number the illustrations with Arabic numerals according to their sequence in the text.
• Use a logical naming convention for your artwork files, and supply a separate listing of the files and the software used.
• Provide all illustrations as separate files and as hardcopy printouts on separate sheets.
• Provide descriptive captions to illustrations separately.
• Produce images near to the desired size of the printed version.

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Regardless of the application used, when your electronic artwork is finalised, please "save as" or convert the images to one of the following formats (Note the resolution requirements for line drawings, halftones, and line/halftone combinations given below.):

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TIFF: Colour or greyscale photographs (halftones): always use a minimum of 300 dpi.
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• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Line drawings

The lettering and symbols, as well as other details, should have proportionate dimensions, so as not to become illegible or unclear after possible reduction; in general, the figures should be designed for a reduction factor of two to three. The degree of reduction will be determined by the Publisher. Illustrations will not be enlarged. Consider the page format of the journal when designing the illustrations.
Do not use any type of shading on computer-generated illustrations.

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Remove non-essential areas of a photograph. Do not mount photographs unless they form part of a composite figure. Where necessary, insert a scale bar in the illustration (not below it), as opposed to giving a magnification factor in the caption.
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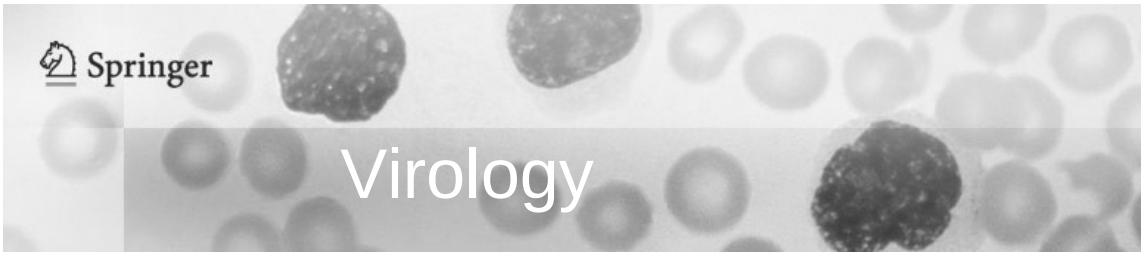
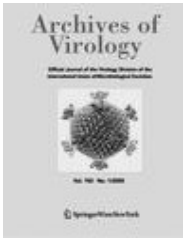
ANEXO B: Normas de publicação do periódico *Archives of Virology*





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Instructions for Authors

Archives of Virology

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Electronic submission substantially reduces the editorial processing and review in times and shortens overall publication times. Please login directly at
www.edmgr.com/avirol and upload your manuscript following the on-screen instructions.
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The review process

Manuscripts are subjected to peer review. Reviews may be done by members of the Advisory Board of Archives of Virology or by qualified ad hoc reviewers. Every effort is made to accelerate this process. Authors receive the written comments of the reviewers, together with notification of acceptance, rejection, or need for modification.

Editorial policy

Manuscripts will be judged on their quality, originality, significance, and interest to the readership. Research work should be described in a form whereby it can be repeated, and its conclusions verified. Articles represent the opinion of the author(s), not that of the members of the Editorial Board or the Publisher.

- Primary publication: Manuscripts should be original, not wholly or in part published or submitted for publication elsewhere. Preliminary disclosure of research findings, such as in a published abstract, thesis, or proceedings of a meeting, is not considered prior publication.
- Authorship: Authorship implies substantial contribution to the overall design, execution, and interpretation of the primary experimental work described in a manuscript. All authors are considered responsible for the entire content of their manuscript.
  Individuals who have supplied virus strains or reagents, or who have critiqued the manuscript, etc., should not be listed as authors; rather they may be recognized in the Acknowledgments section.
- Availability of viruses, mutants/variants, cells: Submission of a manuscript implies that all viruses, novel

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- Biosafety and humane use and care of experimental animals: Submission of a manuscript implies that all local and national biosafety regulations governing exposure of laboratory personnel and the environment to biohazards have been observed.
  Submission of a manuscript also implies that all local and national regulations and guidelines governing the humane use and care of experimental animals have been observed.
- Nucleotide sequence data: Only in exceptional circumstances will long sequences be published. New nucleotide data must be submitted and deposited in the DDBJ/EMBL/GenBank databases and an accession number obtained before the paper can be accepted for publication. Submission to any one of the three collaborating databanks is sufficient to ensure data entry in all. The accession number should be included in the manuscript e.g., as a footnote on the title page: 'Note: Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number(s)----'. If requested, the database will withhold release of data until publication. The most convenient method for submitting sequence data is by World Wide Web:
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  GenBankTM via BankIt: http://www.ncbi.nlm.nih.gov/BankIt/
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  EMBL: EMBL Nucleotide Sequence Submissions, European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB 10 1SD U.K.; telephone: +44 1223 494499; fax: +44 1223 494472; e-mail: datasubs@ebi.ac.uk; web URL: http://www.ebi.ac.uk
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  Encoded amino acid sequences may be presented, if necessary, immediately above or below the first nucleotide of each codon, by using the standard single-letter amino acid code.
- X-ray crystallographic protein structure data: Manuscripts containing new structure determinations (including X-ray amplitudes and phases and derived atomic coordinates) must be accompanied by (an) accession number(s) from an internationally available depository (such as Brookhaven National Laboratory, Upton, NY 11973, U.S.A.). If structure determinations have been submitted to a depository, but an accession number has not yet been assigned, the manuscript can be submitted for review, but it will not be published until (an) accession number(s) is available for insertion into the manuscript.

Form of manuscripts

Only contributions written in English are acceptable; authors who are unsure of proper English usage should have their manuscript checked by someone proficient in the English language, preferable someone who uses English as a primary language. Manuscripts should be concise. Repetition, such as between the Materials and methods section and figure legends, or between the Results section and the Discussion, should be avoided. Avoid jargon and laboratory slang. Good writing requires control of the sequence of words, sentences, paragraphs, and sections. Good writing requires correct use of verb tenses—the past tense should be used throughout in descriptions of particular procedures and observations—the present tense should be used only in making generalizations, such as in noting generally accepted facts, conclusions from prior research, and general conclusions from the work at hand.

Archives of Virology publishes Original Articles, Brief Reports, Brief Reviews, Rapid Communications, Annotated Sequence Records, and Special Issues.

Original Articles: Original articles should not exceed 20 pages when printed. Their content should be arranged as follows:

Title Page: The first page should include the title of the article, the names of the authors, their institutional affiliations (that is, the institution where the work was done), and their full addresses (including all postal codes).

A footnote may be used to cite the present address of any author no longer at the institution where the work was done. This page should also include a running title (not to exceed 60 characters and spaces) and the name and mailing address (incl. e-mail) of the corresponding author.

Summary: The Summary should not exceed 200 words; it should concisely summarize the basic content of the article without presenting experimental details. The Summary should be understandable when published separately by abstracting services. The Summary should be written in the past tense and should not contain references or cryptic abbreviations.

Introduction: The Introduction should supply sufficient background information to establish the context of the present study—it should allow the reader to see the rationale for the present work and to understand and evaluate present results— it should not be too general, nor should it take the form of an exhaustive review of the subject. The Introduction should usually end with one or two sentences that capture the essence of the article: e.g., "In this paper we report the discovery of …"

Materials and methods: The Materials and methods section should provide sufficient information to permit the work to be repeated. For commonly used methods, a brief description (to avoid constant need to refer to previous publications) and citation of a reference are sufficient. New methods should be described completely, giving sources of unusual chemicals, equipment, and supplies. When large numbers of viruses, mutants, etc., are used in a study, a table may be used to identify sources, properties, etc.

Results: The Results section should include the outcome of experiments; extensive interpretations of experimental data should be reserved for the Discussion section. Data should be presented in text, tables, or figures—the same data should not be repeated in two or three forms.

Discussion: The Discussion section should not merely restate the experimental results and immediate conclusions.

It should be constructive, interpretive, analytical, and it should establish the relationship between the results obtained and previously published work. It should note problems, such as conflicts with the ideas and data of others, and it should indicate the value of the results for future research.

Acknowledgments: Acknowledgments of personal assistance and financial support should be stated in concise terms.

Disclaimers: Statements disclaiming governmental or any other type of approval or endorsement will be deleted by the publisher.

References: References, numbered consecutively and arranged in alphabetical order should be listed at the end of the paper as follows:

- a) Journals: Names and initials of all authors, year of publication, complete title of paper, name of journal (abbreviated according to "Index Medicus"), number of volume, first and last page numbers, e.g.,
  1. Bussel RH, Karzo DT (1965) Canine distemper virus in primary and continuous cell lines of human and monkey origin. Arch Ges Virusforsch 17: 183–202
- b) Books: Name(s) of author(s), year of publication, complete title, edition, publisher, place of publication, e.g.,
  2. Goodman LS, Gilman A (1960) The pharmacological basis of therapeutics, 2nd edn. Macmillan, New York or Name(s) of author(s), year of publication, title of chapter or contribution, name(s) of editor(s), if any, title of book, publisher, place of publication, first and last page numbers, the series, if any, plus volume or number in the series, e.g.,
  3. MacPherson I (1966) Malignant transformation and reversion of virus infected cells. In: Kirsten WH (ed) Malignant transformation by viruses. Springer, Berlin Heidelberg New York, pp 1–8 (Recent results in cancer research, vol 6)
- c) Proceedings and Transactions: Name(s) of author(s), year of publication, complete title of article, title of proceedings, if any, name of proceedings, place and year of congress or symposium, publishers, place of publication, first and last page numbers, e.g.,
  4. Barry J, Lefranc G (1962) The occurrence of Gomorinegative neurosecretory material in the diencephalon of Macacus sylvanus L. In: Neurosecretion. Proceedings of the third international conference on neurosecretion, Bristol 1961. Academic Press, New York, pp 209–214

  In the text citations are listed by numbers corresponding to references at the end of the paper.

Figures: Figures should be submitted on separate sheets, numbered in order of mention in the text, and marked on the back with the figure number, the name of the author, and an indication of the top of the figure. The maximum space available on one page 21.5 cm for figures is 13.5 x

Photographs: Photographs should be submitted as glossy prints, sharply focused, with high contrast. Grouping of photographs to fill a page or part of a page is recommended. Photographs should be cropped to include only significant subjects. Make sure that after reduction all labeling will be of the same size and still remain legible (label letters and numbers at least 3 mm high). If a figure is a composite, such as multiple polyacrylamide gels,

the original composite, not just a photograph of the composite, should be provided to the publisher. Electron micrographs and light microscopy photomicrographs should be submitted as direct prints of original negatives, not as manipulated artwork.

Color photographs can be published, but only if authors bear all additional costs of printing.

Line drawings: Graphs, charts, sequences, complicated chemical or mathematical formulas, diagrams, and other drawings should be submitted as originals (with copies with the duplicate manuscript). Drawings should be of professional quality, with lettering large enough to withstand necessary reduction (at least 2 mm high after reduction). Computer graphics and lettering are acceptable, but should be of a quality matching traditional standards.

Figure legends: Legends for figures should be submitted on a separate sheet, numbered consecutively. Legends should provide enough information so that the figure is understandable without frequent reference to the text. Detailed experimental methods should be described in the Materials and Methods section, not in legends.

Tables: Each table should be submitted on a separate sheet; each table should be numbered sequentially, have a descriptive heading, informative column headings, and footnotes that make the table understandable without frequent reference to the text. Authors are advised to follow the design of tables in recent issues. Large, complicated tables, with complex column spacing can be submitted as “camera ready”
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Special Issues: Special issues of Archives of Virology are published to record the proceedings of meetings, symposia, conferences, and congresses on various virologic topics, special issues are also published to record multi-authored treatises and reviews of large, complex virologic topics. In general, special issues are of similar size and page format as the regular issues of Archives of Virology ; the number of pages per issue is limited to 240 pages. The Archives of Virology provides full and flexible publishing and marketing services, in timely fashion. Individuals who are organizing a meeting, symposium, conference, or congress, and individuals who would like to organize the writing and publication of a treatise or large review are invited to communicate directly with the Special Issues Editor, Dr. C. H. Calisher, Arthropod-Borne and Infectious Diseases Laboratory, Colorado State University, Fort Collins, CO 80523, U.S.A. e-mail: calisher@cybercell.net for further information.

Virus nomenclature:
Each virus should be identified at least once, preferably in the Introduction or Materials and Methods section, using formal family, genus, and species terms, and where possible by using a precise strain designation term as developed by an internationally recognized specialty group or culture collection. Please note that the word type

is not used before species designations that include a number. Formal terms used for virus families, genera, and species, should be those approved by the International Committee on Taxonomy of Viruses (ICTV): Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) (2005) Virus Taxonomy. Classification and Nomenclature of Viruses. 8th ICTV Report, Academic Press, Elsevier, 1217 pages. This volume also includes standard abbreviations for species. Once formal taxonomic names have been given in a paper, vernacular terms may be used.

Formal taxonomic nomenclature: In formal taxonomic usage, the first letters of virus order, family, subfamily, genus and species names are capitalized and the terms are printed in italics. Other words in the species name are not capitalized unless they are proper nouns or parts of nouns, for example West Nile virus. In formal usage, the name of the taxon should precede the term for the taxonomic unit; for example: “the family Paramyxoviridae”, “the genus Morbillivirus”. The following represent examples of full formal taxonomic terminology:

1. Order Mononegavirales, family Rhabdoviridae, genus Lyssavirus, Species Rabies virus.
2. Family Poxviridae, subfamily Chordopoxvirinae, genus Orthopoxvirus, species Vaccinia virus.
3. Family Picornaviridae, genus Enterovirus, species Poliovirus.
4. Family Bunyaviridae, genus Tospovirus, species Tomato spotted wilt virus.

Vernacular taxonomic nomenclature: In formal vernacular usage, virus order, family, subfamily, genus and species names are written in lower case Roman script; they are not capitalized, nor are they printed in italics or underlined. In informal usage, the name of the taxon should not include the formal suffix, and the name of the taxon should follow the term for the taxonomic unit; for example “the picornavirus family”, “the enterovirus genus”.

One particular source of ambiguity in vernacular nomenclature lies in the common use of the same root terms in formal family, genus or species names. Imprecision stems from not being able to easily identify in vernacular usage which hierarchical level is being cited.

For example, the vernacular name “paramyxovirus” might refer to the familly Paramyxoviridae, the subfamily Paramyxovirinae, or one species in the genus Respirovirus, such as Human parainfluenza virus 1. The solution in vernacular usage is to avoid “jumping” hierarchical levels and to add taxon identification wherever needed. For example, when citing the taxonomic placement of Human parainfluenza virus 1, taxon identification should always be added: “Human parainfluenza virus 1 is a species in the genus Respirovirus, family Paramyxoviridae.” In this example, as is usually the case, adding the information that this virus is also a member of the subfamily Paramyxovirinae and the order Mononegavirales is unnecessary.

It should be stressed that italics and capital letters must be used only when referring to taxonomic categories like species, genera and families. When referring to the virus being studied rather than to the taxonomic group the virus belongs to, the virus name is written in lower case Roman script without capitals, for instance measles virus or tomato chlorosis virus. It is incorrect to write that the species Tobacco mosaic virus has been sequenced or has been isolated from a host plant, since it is only the virus itself, tobacco mosaic virus, that can be handled in this way and has a sequence. Virus species, like genera or families, are man-made taxonomic constructions and do not have hosts, vectors or sequences. On the other hand, it is correct to write that a member (i.e. a virus), a strain or an isolate of the species Tobacco mosaic virus has been isolated or was sequenced. The use of italics when referring to the name of a species signals that it has the status of an official species recognized by the ICTV.

The 8th ICTV Report (Fauquet et al 2005, Academic Press) should be consulted to ascertain which names have been approved as official species names. Tentative species names not yet approved by the ICTV are not written in italics.

Nomenclature of bacteria: Binary names, consisting of a genus and species term (e.g., Escherichia coli), should be used for all bacteria. After the first usage, the genus term should be abbreviated (e.g., Escherichia coli). All taxonomic terms, including genus, species, and subspecies are printed in italics; strain designations are not.

Genetic nomenclature: Where appropriate for viral genetic systems (e.g., phenotypes, genotypes, wild-type alleles), the nomenclature recommendations of Demerec et al. should be used: Demerec M, Adelberg EA, Clark AJ et al (1966) A proposal for a uniform nomenclature in bacterial genetics. Genetics 54: 61–76.

Chemical and biochemical nomenclature: The names used for chemical/biochemical compounds should be those recommended in Chemical Abstracts and its indices (Chemical Abstracts Service, The Ohio State University, Columbus, Ohio, U.S.A.), Biochemical Nomenclature and Related Documents (The Biochemical Society, London, U.K.), and the Instructions to Authors of leading biochemistry journals. For enzymes, use terms recommended by The International Union of Biochemistry (1984) Enzyme nomenclature. Academic Press, New York.

Restriction endonucleases: Nomenclature for restriction endonucleases should follow standard convention: Roberts RJ (1977) Restriction endonucleases. In: Bukhari AQI, Shapiro JA, Adhya SL (eds) DNA insertion

elements, plasmids, and episomes. Cold Spring Harbor Laboratory, New York, pp 757–768.
Nucleotide sequence data: The sequencing strategy employed should be described, and the sequence itself submitted in the form of “camera ready” copy, following standard conventions of the International Union of Biochemistry.

Abbreviations
Abbreviations should aid the reader; therefore, their use should be limited. Generally, abbreviations should not be used for terms appearing less than five times in the article. Where abbreviations are used, they should follow the usage established by Chemical Abstracts Service Source Index (Chemical Abstracts Service, The Ohio State University, Columbus, Ohio, U.S.A.).
Standard abbreviations: The following abbreviations do not require definition: DNA; RNA; cDNA; cRNA; rRNA; tRNA; mRNA; DNase; RNase; AMP; ADP; ATP; GTP; ATPase; dGTPase; NAD; NADH; NADP; poly(A); poly(dT); oligo(dT); UV; PFU; CFU; Tris; DEAE; EDTA; HeLa; Vero; MEM; and similarly common terms.
Numerical data. Units of measurement: Standard metric units are used for length, weight, and volume. For these units and for molarity, use standard terms: m, µ, n, and p, for 10^{-3}, 10^{-6}, 10^{-9}, and 10^{-12}, respectively. Use the term k for 10^{3}. Avoid compound terms such as mµ or µµ. Avoid the ambiguous term ppm (instead use µg⁄ml or µg/g). Units of temperature are written as 37°C or 324 K. See standard references for reporting units of illumination, energy, frequency, pressure, etc.
Molecular mass: When indicating the mass of viruses, ribosomes, and other biologically complex entities containing different kinds of molecules, the term molecular mass (M_r), not molecular weight, should be used. (M_r is a dimensionless number representing the ratio of the mass of an entity to one-twelfth the mass of an atom of ^{12} C.) When indicating the mass of proteins, carbohydrates, and other complex molecules, the term M_r; is also preferred, but molecular weight may be used. It is acceptable, but not necessary, to use the mass unit Dalton with the term M_r , but not with the term molecular weight. For example, it is preferred to state that the M_r of the poliovirus virion is 8.58 x 10^{6}; and that the M_r of poliovirus protein VP- 1is 33,521.
Isotopically labeled compounds: For simple molecules, isotopic labeling is indicated in the chemical formula or name (e.g., $^{14}CO_2$, H_2 $^{35}SO_4$, $^{-14}$C-amino acids, ^{131}I-labeled protein). For complex molecules, the symbol for the isotope introduced is placed in square brackets directly preceding the part of the name that describes the labeled entity (e.g., [^{14}C]urea, SV 40 [^{32}P]DNA, [a-^{14}C]lysine). See Instructions to Authors of leading biochemistry journals for further details.

Patient identification
When virus isolates are derived from patients in clinical studies, do not identify them by using patients’ names or initials, even as part of a strain designation. Do not use hospital identifiers. Instead, use confidentially coded terms. Note: established designations of some viruses and cells that represent patient initials are acceptable—JC virus, BK virus, HeLa cells, etc. Do not use patient group identifiers pertaining to race, address, occupation, etc., unless relevant to the study.

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